

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

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| Serial No.: | 09/436,347 | Group Art Unit: | 1643 |
| Confirmation No.: | 6491 | Examiner: | A.M. Harris |
| Filed: | 9 November 1999 | | |
| Inventor: | Christine A. WHITE <i>et al.</i> | | |
| For: | Treatment of Chronic Lymphocytic Leukemia using Anti-CD20 Antibodies (as amended) | | |

SECOND DECLARATION OF DAVID P. SCHENKEIN, M.D. UNDER 37 C.F.R. § 1.132

1. I am Senior Vice President, Clinical Hematology/Oncology at Genentech, Inc. (licensee of the above patent application). Genentech, a licensee of patent application No. 09/436,347 ("the '347 application"), co-promotes RITUXAN® (rituximab), a therapeutic CD20 antibody, in the United States together with Biogen Idec Inc., the owner of the patent application.

2. I have previously provided a declaration in this case. As I explained in that declaration, I specialize in the field of hematologic malignancies, and was actively treating chronic lymphocytic leukemia (CLL) patients in November of 1998 when the '347 application was filed. My credentials and background are essentially as I described them in my previous declaration.

3. I have reviewed the final Office action of February 19, 2009 ("Final Action"). I have also reviewed the following patents and publications cited in the Final Action:
 - RITUXAN® (rituximab) package insert dated November, 1997;
 - U.S. Patent No. 5,843,398 ("Kaminski '398 patent") and U.S. Patent No. 6,090,365 ("Kaminski '365 patent");
 - U.S. Patent No. 5,736,137 ("Anderson patent");
 - U.S. Patent Application No. 2003/0018014A1 ("Lerner");
 - Stenbygaard *et al.*, Breast Cancer Research and Treatment 25:57-63 (1993) ("Stenbygaard"); and
 - McLaughlin *et al.*, J. Clin. Oncology, 16(8):2825-2883 (Aug. 1998) ("McLaughlin").

4. As I have previously explained, in 1998, I was a practicing physician with extensive experience treating CLL patients. I believe the opinions in my declarations are representative of the opinions that a person of ordinary skill in the art in the field of this invention would have had at that time.
5. At page 6 of the Final Action, the Examiner states that I did not provide in my earlier declaration "sufficient evidence teaching the immunotherapeutic mechanisms, host effector functions and receptor binding affinity of the CD20 antibody would differ between the two diseases, resulting in different antitumor mechanisms and significant differences in the impact of the therapy."
6. In paragraphs 20 and 29 of my earlier declaration, I explained that the CD20 antigen density on CLL cells was known to be lower than that on non-Hodgkin's lymphoma (NHL) cells, and that this unique feature of neoplastic CLL cells reduces susceptibility of these cells to cell killing by an anti-CD20 antibody.
7. Perhaps in response to these observations, the Examiner, at page 5 of the Final Action, states that the Kaminski '398 patent "plainly and clearly teaches the effectiveness of CD20 therapy for a B cell cancer and the high expression of CD20 antigen on CLL (more than 95% expression on patients with CLL)." The Examiner cites column 8, lines 9-16 of the Kaminski '398 patent to support this conclusion. The Examiner's characterization of this passage of the patent is scientifically incorrect.
8. By 1998, it was known that neoplastic B cells from CLL patients exhibit a *low density* of CD20 antigen. See, Almasri *et al.*, *Am. J. Hematol.* 40:249-263 (1992) (provided with my earlier declaration). In other words, while CD20 antigen can be detected on most neoplastic cells isolated from CLL patients, the number of antigens expressed by and accessible on the cell surface of each cell is *low* compared to other types of normal and abnormal B-cells. This low density or "dim" CD20 antigen expression was known to be a unique feature of CLL cells. See, column 1 on page 263 of Almasri *et al.*

9. By contrast, in 1998, it was known that neoplastic B-cells from patients with NHL exhibit a high density of CD20 antigen. See, for example Table 1 on page 260 of Almasri *et al.* In addition, it was believed that antibody dependent cellular cytotoxicity (ADCC) and complement dependent cytotoxicity (CDC) were important mechanisms for the *in vivo* response of anti-CD20 antibodies in treatment of NHL patients, and that the “high-density expression” of CD20 on NHL cells was necessary for efficient lysis of neoplastic B-cells from NHL patients by CDC and ADCC mediated by anti-CD20 antibodies. See, Anderson *et al.*, *Biochem. Soc. Trans.* 25: 705-708 at column 1 on page 706 (1997) (attached as Exhibit A).
10. The contrasting *low* level of CD20 expression on CLL cells therefore would have been one important reason why an oncologist in 1998 would have doubted that anti-CD20 antibodies could be used to effectively treat CLL patients. In particular, the low density of expression was believed to prevent anti-CD20 antibodies from mediating CDC and ADCC in neoplastic cells from CLL patients.
11. This view is reflected in later publications. For example, Farag *et al.* showed that the reduced levels of expression of CD20 antigen on neoplastic cells from CLL patients significantly reduces the efficacy of anti-CD20 antibodies in mediating ADCC in CLL. See, for example, Farag *et al.*, *Blood* 103(4): 1472-1474 (2004) (attached as Exhibit B), at page 1474, column 1. Farag *et al.* explains that CDC is less likely to occur with CLL cells because those cells “often dimly express CD20”. See, Farag *et al.*, page 1474, column 2. Farag *et al.* also points out that overexpression of CD55 and CD59 on B-cells prevents CDC, and that that CLL cells have been shown to overexpress these two complement inhibitors. See, Farag *et al.*, column 2 on page 1474.
12. Golay *et al.* also explains that low levels of CD20 expression reduce susceptibility of CLL cells to an anti-CD20 antibody and complement. See, Golay *et al.*, *Blood* 98(12): 3383-3389, 3385 (2001) (attached as Exhibit C). Golay *et al.* explain that CDC was thought to be an important mechanism of action of an anti-CD20 antibody and that the degree of the lytic response (CDC activity)

depended on the level of expression of CD20 on the cells. See, Golay *et al.*, abstract and Fig. 8 on page 3388.

13. Therefore, even though greater than 95% of CLL patients express *some* CD20 antigen on their neoplastic cells, this would not have provided an oncologist in 1998 with a legitimate scientific basis for believing that an anti-CD20 antibody could be used to effectively treat patients with CLL. Instead, in 1998, an oncologist would have believed that the low density of expression of CD20 would have been the relevant factor, because it would render these neoplastic cells in the CLL patient not susceptible to ADCC and CDC – two mechanisms of action believed to be important to the way that anti-CD20 antibodies provided therapeutic effectiveness against neoplastic cells in NHL patients.
14. My earlier declaration also explained that another important difference between CLL and NHL was that patients with CLL have a much higher circulating tumor burden than patients with NHL. See, paragraphs 22 and 30 of my first declaration. The Examiner does not address this important difference between CLL and NHL in the Final Action.
15. The high tumor burden in CLL patients would have raised additional questions in the mind of an oncologist working in this field in 1998 about whether an anti-CD20 antibody would be effective in providing therapeutic benefits in a CLL patient. For example, an oncologist would have been concerned that a CLL patient could not mount an effective immune response against the neoplastic CLL cells in the patient following administration of the anti-CD20 antibody due to the high tumor volume.
16. Again, concerns that were held in 1998 are reflected in the scientific literature published later. For example, Kennedy *et al. J. Immunol.* 172(5): 3280-3288 (2004), copy attached as Exhibit D, identified a number of important potential issues that had been identified concerning the treatment of CLL patients with high tumor cell burdens with an anti-CD20 antibody, including: (i) that the treatment would consume so much complement that the ability of the antibody to

promote CDC could be compromised, (ii) that the capacity of the mononuclear phagocytic system (MPS) to remove IgG-opsonized cells might be exceeded at high cell counts, and (iii) that Fcγ receptor mediated rearrangement and capping of antibody-antigen complexes on the surface of B-cells might lead to removal of the complexes, thus allowing cells to escape.

17. In other words, in 1998, there were a number of significant scientific reasons why an oncologist would not have expected that an anti-CD20 antibody would provide positive clinical benefits in treating CLL patients despite the evidence of successful use of such antibodies in treating NHL patients as in the cited references.
18. The Examiner also incorrectly suggests at page 5 of the Final Action that Example IV in the Kaminski '398 patent discloses that an "unlabeled anti-CD20 antibody is used in combination with chemotherapy." The Examiner's characterization of the Kaminski '398 patent and Example IV are inaccurate.
19. The treatment methods described in the Kaminski '398 patent use monoclonal antibodies that recognize tumor-associated antigens to *deliver radioisotopes* to tumor cells (column 1, lines 46-52 of the Kaminski '398 patent). Because it was known that β-emitters such as ⁹⁰Y or ¹³¹I had ranges of emission resulting in a "bystander effect," cells adjacent to the cell bound by the radiolabeled antibody could be killed by the radiolabel. This is discussed at column 9, lines 23-44 of the Kaminski patent. In these methods, both the particular tumor cell antigen and the capacity or nature of binding of the antibody to it become relatively unimportant – as long as the radiolabeled antibody can bind to cells in the proximity of the tumor mass, the radioisotope bound to the radiolabeled antibody can deliver its therapeutic effect.
20. Example IV in columns 35-36 of the Kaminski '398 patent discusses "sensitization of lymphoma cells." Initially, it is important to recognize that the hypothesis described in this paragraph is not focused on CLL cells, but instead is based on the experiences of the Kaminski inventors with lymphoma cells. It also

is not supported by any experimental data, does not discuss treatment of any patient, much less a CLL patient, and provides no details about timing, dosages or other aspects of any particular possible therapeutic regimen.

21. Example IV hypothesizes that there might be synergism in the induction of apoptosis in lymphoma cells occurring as a consequence of the concurrent binding of an anti-CD20 antibody to a B-cell and the delivery to that B-cell of a dose of irradiation via the radiolabeled antibody. See, Kaminski '398 patent at column 35, lines 37-38. Based on this hypothesis, Kaminski suggests that it might be possible to achieve a similar synergism through the administration of a non-radiolabeled antibody to CD20 plus "a second antibody, directed against a different antigen than CD20, that is conjugated to a radionucleotide." As Kaminski suggests, this "would provide the same synergistic second insult to the tumor cell as is provided by an anti-CD20 radioimmunoconjugate." See, Kaminski '398 patent at column 35, lines 42 to 51. In other words, to achieve the desired effect, this process would employ an non-radiolabeled anti-CD20 antibody, which would not have an independent therapeutic effect, with a second radiolabeled antibody against a different B-cell antigen, which would irradiate the cells.
22. The Kaminski '398 patent then refers to other theoretical approaches for potentially achieving "synergism in the induction of apoptosis" in lymphoma. These approaches include external beam irradiation (column 35, lines 53-60) or administering a chemotherapeutic agent (paragraph spanning columns 35 to 36). These additional passages do not indicate that a non-radiolabeled anti-CD20 antibody should be combined with these other treatments.
23. I do not believe an oncologist would have used the information provided in this Example or elsewhere in the Kaminski patent to devise a new treatment method for CLL in 1998 using non-radiolabeled anti-CD20 antibodies. In 1998, as I have explained, an oncologist would question whether a significant ADCC or CDC response could be induced in a CLL patient by administration of a non-radiolabeled anti-CD20 antibody due to the low levels of CD20 expression by

neoplastic cells in CLL patients, and the high volume of those cells in the patient. This would have, at a minimum, raised serious questions about whether any positive clinical benefits would be seen in CLL patients given a non-radiolabeled anti-CD20 antibody. None of these issues are resolved by the information provided in Example IV or elsewhere in the Kaminski '398 patent, as the approaches described in the Kaminski patent depend on use of radiolabeled antibody that kills targeted cells using a fundamentally different mechanism.

24. Because of this, even if the Kaminski '398 patent had shown that *radiolabeled* anti-CD20 antibodies provided some positive clinical benefits in *CLL* patients (which it did not), this fact would not have led an oncologist familiar with CLL to believe that administration of *non-radiolabeled* anti-CD20 antibody would be effective in treating CLL. Instead, given the emphasis throughout the Kaminski '398 patent on *radioimmunotherapy* and the observations in the patent about the "limited efficacy of unmodified antibodies" (column 2, lines 20-22), I believe the patent actually would have taught away from the idea of treating CLL patients with non-radiolabeled anti-CD20 antibodies as required by claims 29, 55 and 97 of the '347 application.
25. At page 5 of the Final Action, the Examiner states that there "seems to be no factual evidence presented suggestive of failure of treatment of CLL in a patient." I disagree. I believe I identified a substantial amount of factual evidence in my earlier declaration that shows why an oncologist would not believe that treatment of CLL patients using an anti-CD20 antibody would be therapeutically effective based on the references cited in the Final Action. See, for example, paragraphs 6-15 and 28-33 of my earlier declaration.
26. In addition, I believe my personal experiences from 1998 are also relevant. In 1998, I became aware of the use of the anti-CD20 antibody rituximab to treat NHL. Despite this, I did not use rituximab to treat CLL patients under my care. I did not do this because I did not think that rituximab would provide positive clinical benefits given the significant differences between the diseases and the

unique characteristics of neoplastic CLL cells. I also had major concerns about serious adverse side effects related to the very high tumor cell burdens in CLL patients.

27. The publications I cited in my earlier declaration and in this declaration also provide the evidence that the Examiner has requested. These publications document the low density of CD20 expression on cells from CLL patients and the high circulating tumor burden in CLL patients, which support my opinion that oncologists would have believed in 1998 that an anti-CD20 antibody-based treatment regimen for CLL patients would not have provided positive clinical benefits to CLL patients.
28. In addition to these reasons, I believe there is additional scientific evidence in the literature showing that administration of anti-CD20 antibodies did not provide positive clinical benefits for CLL patients. In particular, I note that Jensen *et al.* *Ann. Hematol.* 77: 89-91 (1998) (attached as Exhibit E, and previously provided to the Examiner) presents evidence of a failed attempt to treat a CLL patient using rituximab.
29. Jensen *et al.*, reports administration of rituximab to a patient with CLL at a dose of 375mg/m². After administration of the first infusion of rituximab, the patient suffered "severe side effects," which the authors attribute to "rapid tumor lysis syndrome." See, Jensen *et al.*, column 1 on page 89. While three further infusions were administered "without clinical problems," the paper reports that the treatment was *ineffective* – the patient showed signs of progressive disease at 3 weeks requiring salvage chemotherapy. See, Jensen *et al.*, at page 90, column 2.
30. The serious side effect of rapid tumor cell lysis reported in Jensen *et al.* was the basis of one of the concerns I had in 1998 about administration of an anti-CD20 antibody to a patient with a high circulating tumor burden. As Jensen *et al.* point out, "physicians must be aware of this hitherto unreported phenomenon in patients with high CD20-positive blood counts." See, Jensen *et al.*, at page 89 (summary).

31. I also note that the abstract of Jensen *et al.* explains that earlier trials in follicular lymphoma excluded patients with lymphocytes $> 5 \times 10^9/L$ (such high tumor burdens being characteristic of CLL). Given the serious side effects and lack of positive clinical benefits in CLL patients as reported by Jensen *et al.*, this suggests that the decision to exclude patients with lymphocyte counts $> 5 \times 10^9/L$ in McLaughlin *et al.* was not made for the simple purpose of "streamlining the patient population for testing" as the Examiner suggests at page 4 of the Final Action. Instead, a more plausible explanation is that the authors believed that administration of anti-CD20 antibodies to CLL patients would place them at risk of serious side effects with no prospect for positive clinical benefits.
32. Jensen *et al.* also refers to minor "clinical side effects" in six additional CLL patients treated with rituximab. The paper, however, does not report that rituximab was clinically active in any of these patients. In view of the ineffectiveness in the patient that is the focus of the Jensen *et al.* report, I believe an oncologist would have concluded that the other patients given rituximab, likewise, did not achieve a positive clinical benefit from the treatment.
33. I understand that all of the pending claims of the '347 application require administration of an anti-CD20 antibody "*in an amount effective to treat the CLL.*" This means to me that the treatment must result in a positive clinical benefit to the CLL patient. I do not believe a method that results in the continued progression of the CLL disease in the patient would be considered to be "effective to treat CLL" by an oncologist. In addition, a method that induces only an undesirable and life-threatening condition in the CLL patient, such as rapid tumor cell lysis, would not qualify as being a method that is "effective to treat the CLL."
34. My opinion of what this expression in the claims would convey to an oncologist is consistent with the specification of the '347 application, which refers to treatment methods that result in, for example, demonstrated efficacy with minimal infusion-related toxicity (page 8, paragraph 0320), overall response rate (ORR), complete responses (CR), partial responses (PR), improved median time to progression or

improved duration of response (page 9, paragraph 0340 as well as page 14, paragraph 0440), or remission upon treatment (page 11, paragraph 0370).

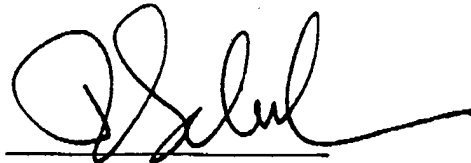
35. The treatment of CLL patients using anti-CD20 antibodies is an important medical advance addressing a long felt need in the treatment of CLL. CLL is the most common form of leukemia in adults, has an incidence of approximately 15,000 cases per year in the United States, and annually causes about 4,500 deaths. In 1998, the clinical prognosis for an individual with CLL was bleak, and that outcome in the treatment of CLL had improved little over the preceding 30 years. See, Catovsky *et al. European Journal of Cancer* 31A Nos. 13/14: 2146-2154 (1995) (copy attached as Exhibit F).
36. The treatment regimes described and claimed in the '347 application are effective in treating CLL, in both previously untreated CLL patients (see, for example, Halleck *et al.*, *Blood* 112(11), Abstract 325 (2008), copy attached as Exhibit G) and in CLL patients in which chemotherapy has failed to slow the progression of the disease (see, for example, Robak *et al. Blood* 112(11): Abstract LBA-1 (2008), copy attached as Exhibit H). Halleck *et al.* and Robak *et al.* report phase III clinical trials confirming that CLL can be treated in human patients by administering an anti-CD20 antibody to the patients in an amount effective to treat the CLL as disclosed and claimed in the '347 application. Complete responses, partial responses, and improved median progression-free survival confirm the therapeutic efficacy of this treatment regimen. The trials did not include treatment with a radiolabeled antibody or use of radiation in conjunction with the therapeutic anti-CD20 antibody. The data from these trials demonstrates the effectiveness of the claimed methods of treating CLL patients, and establishes that the claimed treatment methods address the long felt need for a safe and effective way to treat CLL patients.
37. In addition, at the time of the invention, the most viable method of treatment of CLL patients was using fludarabine. See Catovsky *et al.* While fludarabine is useful in treating many patients, there are a significant number of patients who

do not respond to fludarabine. These fludarabine-refractory patients had no viable alternative treatment options in 1998. Again, the treatment method being claimed in the '347 application responds to the long felt need for a viable way to treat these CLL patients.

38. The CLL treatment methods disclosed and claimed in the '347 application was approved the European Medicines Agency (EMA) earlier this year, and is expected to obtain marketing approval by the Food and Drug Administration (FDA) later this year. CLL treatment with an anti-CD20 antibody is considered by hematology oncologists to be a significant medical breakthrough that will "change practice." See, for example: www.roche.com/media/media_releases/med-cor-2009-02-27.htm, and www.medpagetoday.com/MeetingCoverage/ASHHematology/12054, copies attached as Exhibits I and J, respectively. In my opinion, the methods of CLL treatment in the '347 application will become the standard of care for CLL patients in the future.

* * *

I declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of any patent granted on this application.



David P. Schenkein, M.D.

Date: 05/05/09

Targeting Cytotoxic Immunotherapy

BSI Parallel Session Organized and Edited by P. Amlot (Department of Immunology, Royal Free Hospital).
660th Meeting. Joint Congress with the British Society for Immunology, held at Harrogate,
10-13 December 1996.

Targeted anti-cancer therapy using rituximab, a chimaeric anti-CD20 antibody (IDEC-C2B8) in the treatment of non-Hodgkin's B-cell lymphoma

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Introduction

Rituximab (formerly IDEC-C2B8) is a genetically engineered chimaeric mouse/human monoclonal antibody directed against the CD20 antigen on normal and malignant B-cells. Rituximab is a high-affinity antibody with engineered human immune effector functions designed for promoting tumour cell lysis in patients with non-Hodgkin's B-cell lymphoma (NHL). Rituximab binds antigen and, in turn, binds C1q and activates complement-dependent cytotoxicity (CDC). The antibody also binds strongly to human Fc receptors and thus mediates cell killing through antibody-dependent cellular toxicity (ADCC) and other accessory cell mechanisms.

Preclinical studies have shown that the chimaeric antibody selectively depletes B-cells in peripheral blood and lymph nodes of macaque monkeys [1]. In a phase I clinical trial, 15 patients with relapsed low-grade B-cell lymphoma were treated with a single dose of antibody ranging from 10 to 500 mg/m² administered intravenously [2]. Tumour regressions occurred in six of nine patients who received doses greater than 100 mg/m².

Key factors in successful cytotoxic antibody targeting

The ability of a therapeutic antibody to mediate a targeted cytotoxic response without damage to normal tissues depends on several factors. These factors include the nature of the antigen, the extent of distribution or expression on normal

and diseased tissues, and the ability of the antibody to access the tumour mass. On binding to the target, the antibody must recruit host effector mechanisms that are provided by genetically engineered human gamma 1 constant region. In addition, the antibody must not be immunogenic, to avoid generating neutralizing antibodies in the patient, which could be accomplished by engineering chimaeric, humanized or primate-derived antibodies. The antigen should remain associated with the target, avoiding shedding or internalizing on binding of the antibody. Such criteria were met in the development of rituximab for clinical use in the treatment of B-cell lymphoma.

Defining target antigen characteristics

The IDEC-C2B8 antibody recognizes the human B-lymphocyte-restricted differentiation antigen, CD20. The antigen is expressed at high density on both normal and malignant B-cells [3,4]. CD20 is a cell-surface non-glycosylated hydrophobic phosphoprotein of 35 kDa [4-10], and is expressed beginning with early pre-B-cell development. The pluripotent stem cells and pro-B-cells are CD20-negative, as are plasma cells. The fact that CD20 is expressed on normal B-cells is of little consequence, as there remains a renewable source of B-cells in the CD20-negative precursor population. The CD20 antigen target provides an added benefit, as it restricts the antibody binding to a relatively small 41 amino acid extracellular domain [11]. The small extracellular domain provides an ideal target, placing the antibody in close proximity to the membrane, which maximizes the efficiency of effector mechanisms. The strong hydrophobic

Abbreviations used: ADCC, antibody-dependent cellular toxicity; CDC, complement-dependent cytotoxicity; NHL, non-Hodgkin's lymphoma.

influence of the transmembrane segment keeps the target tightly associated with the intact membrane, with no appreciable shedding. The high-density expression renders it an ideal target for efficient cell lysis by CDC and ADCC mechanisms.

Essential elements of targeting antibodies

In addition to antigen properties, an equally important feature of effective antibody targeting is the ability of the antibody to promote cell killing through efficient effector mechanisms. The ability of rituximab to utilize normal immune system cytotoxic functions was tested *in vitro* in CDC and ADCC assays. Target cell killing through complement activation (CDC) and by binding to Fc receptors on human effector killer cells are proven mechanisms by which engineered antibodies deplete antigen-positive cells [12]. Figure 1 shows ADCC and CDC cell-killing results with rituximab. Rituximab antibody was effective in the killing of CD20-positive SB cells (an Epstein-Barr virus-transformed cell line) in the presence of rabbit complement, whereas a control IgG₁ construct containing the same anti-CD20 variable region was not.

ADCC is a mechanism by which low-affinity FcRIII-positive effector cells can lyse target cells with surface-bound antibody. Effector cells that mediate this lysis include granulocytes, macrophages and natural killer cells. Most experiments give more than 70% cell killing with IDEC-

C2B8, compared with insignificant killing contributed by isotype-matched control.

Engineering of effector function and B-cell depletion *in vivo*

Previous studies with the IDEC-C2B8 antibody have shown that doses as low as 0.4 mg/kg effectively deplete more than 95% of peripheral blood B cells in cynomolgus monkeys [1]. The importance of engineering antibodies with strong effector capabilities is further recognized when actual depletion studies on modified forms of IDEC-C2B8 are done *in vivo*. In a small *in vivo* primate study with cynomolgus monkeys, two forms of engineered IDEC-C2B8 were examined for depleting ability. Fully functional IDEC-C2B8 (IgG₁, K) was administered to a single monkey intravenously (i.v.) in a 2 mg/kg dose. A comparable amount of chimaeric C2B8 antibody engineered with an IgG₁ heavy chain isotype, which also included additional heavy chain substitutions [13,14], C2B8PE (anti-CD20, IgG4PE), was given to two monkeys. After 2 days, blood samples were taken and analysed by flow cytometry to determine the relative percentages of B-cells remaining in circulation. The results shown in Figure 2 show nearly complete depletion of peripheral blood B-cells in 2 days by the control IgG₁ depleting construct, confirming earlier studies [1]. In contrast, the average percentages compared with pre-existing B-cells present in the other two test animals, which

Figure 1

IDEC-C2B8 (rituximab) activates CDC and ADCC in the killing *in vitro* of CD-20-positive target cells

Rituximab (6.25 µg/ml) was added to wells containing Cr-labelled CD20 positive SB cells [12]. Target cells were lysed in the presence of rabbit complement (CDC) as well as human complement or human peripheral blood mononuclear cells activated overnight with interleukin 2 (ADCC). Control IgG used in the CDC assay was an IgG₁ construct of IDEC-C2B8. Control IgG used in the ADCC assay was a chimaeric isotype-matched IgG₁, which does not recognize CD20.

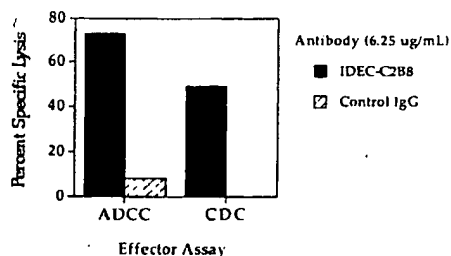
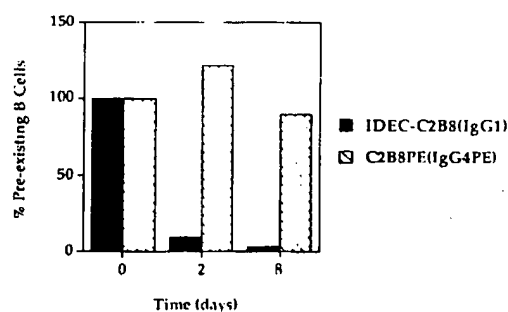


Figure 2

Influence of Fc binding domains on B-cell depletion *in vivo* by engineered chimaeric antibodies (rituximab) and C2B8-PE

Data are expressed as a percentage of pre-existing B-cells obtained from an average of two bloods before administration of test antibodies. Depletion values shown for the IgG₁-treated animals are averaged from the percentages obtained in two animals.



received the modified C2B8-PE antibody, reflect little or no depletion of B-cells.

Gene expression and manufacturing

Depletion of cells in peripheral blood is generally achieved with low doses of antibodies, because high plasma levels of antibody are easily obtained. In B-cell lymphoma, sufficient amounts of the targeting antibody must be administered to ensure saturation of targets within the lymphatic and bone marrow compartments. Frequently, tumour burdens might be exceptionally high, requiring high doses of the antibody. Highly efficient gene expression and manufacturing technology are of major importance for producing sufficient amounts of clinical grade antibody.

For the manufacture of commercial quantities of antibody, high-level expression vectors were employed as described elsewhere [1,15]. Light- and heavy-chain variable regions were cloned, sequenced and placed into the cassette vectors. Chinese hamster ovary cells adapted for rapid growth in serum-free media were electroporated and amplified in methotrexate, resulting in the isolation of a highly productive manufacturing cell line. Under optimal conditions these cell lines are capable of producing in excess of 600 mg/l in 6 days, making it possible to produce large quantities of a therapeutic protein at reasonable cost.

Results of phase II multidose clinical trial with IDEC-C2B8 in patients with B-cell lymphoma

Preclinical studies *in vitro* and *in vivo* with rituximab have provided support for use of the antibody in the clinic [1]. In a phase I single-dose clinical trial [2], the antibody was shown to be safe, well tolerated and efficacious with relatively mild adverse events. In a subsequent phase I/II

multidose trial, patients with relapsed low-grade or follicular NHL received intravenous infusions of 375 mg/m² given weekly for a total of four doses. Infusion of multiple doses provided evidence that the infusion-related adverse events (low-grade fever, flu-like symptoms not as severe as OKT3) were observed with the first infusion and would significantly decrease with subsequent treatments, primarily owing to the clearance of B-cells in peripheral blood during the initial infusion. The clinical response of patients in the phase II portion of the study is shown in Table 1. Three of 34 evaluable patients (9%) had a complete response and 14 of 34 evaluable patients (41%) had a partial response for an overall response rate of 50%; 11 patients (32%) had stable disease and six had progressive disease (2%).

The median time to onset of response for the 17 responders was 50 days (range 7–112 days), and the median time to progression was 10.2 months, with five patients exceeding 20 months. The median duration of response was 8.6 months. At the time of this report, responses are continuing in three patients at 24.8, 27.0 and 27.9 months.

Discussion

Therapeutic alternatives for relapsed patients with low-grade or follicular lymphoma are less than satisfactory. Treatment is usually required because of progressive disease, marrow invasion affecting blood counts, symptomatic bulky nodal disease, B symptoms or disease that threatens critical organs. The available therapies are often accompanied by significant toxicity, and most of them require administration over a prolonged period of several months. In contrast, rituximab represents a unique form of outpatient treatment that can be administered over 22 days with a

Table 1 Clinical response

Phase II: summary of clinical response

Abbreviations: n, number of patients; CR, complete response; PR, partial response; CI, confidence interval. Evaluable patients were those who had low-grade or follicular lymphoma and completed four study treatments

| Patient group | n | CR (%) | PR (%) | CR and PR (%) | 95% CI (CR and PR) (%) |
|-----------------|----|--------|---------|---------------|------------------------|
| Intent to treat | 37 | 3 (8) | 14 (38) | 17 (46) | 30–62 |
| Evaluable | 34 | 3 (9) | 14 (41) | 17 (50) | 33–67 |

novel mechanism of action and a favourable toxicity profile.

The CD20 molecule expressed on neoplastic B-cells provides an excellent target for the therapy of B-cell lymphomas. The studies described in this report establish the importance of effector functions for targeting of antibodies *in vivo* to accessible cell targets. B-cell lymphoma is especially suitable as a target for antibody-mediated therapy because of localization within the accessible lymphatic system and the sensitivity of haemopoietic tumours to lysis by immune effector mechanisms. The CD20 antigen is normally not shed from the cell surface, and CD20 escape mutants are not detected. The CD20 antigen does not internalize after binding by the antibody, thus providing an efficient target for host effector mechanisms.

Rituximab can be given on an outpatient basis with optional premedication. Treatment is brief and is completed in 22 days. In the phase II multidose trial the majority of patients completed all four infusions. Responses have occurred in patients with bulky and non-bulky disease and in patients who had previously received multiple chemotherapy and biological regimens including autologous bone marrow transplants. Indeed, retrospective examination with various prognostic indices of patients treated in this trial indicates that they represent a relatively high-risk group in which low response rates, brief response durations and a short median survival would be expected. Adverse events occurred primarily during the first infusion and were notably diminished and manageable with subsequent infusions. Haemopoietic side effects were mild and transient, in sharp contrast with those seen with traditional chemotherapy and the newer experimental single agents. Quantifiable anti-globulin responses did not occur (one patient had a detectable but not quantifiable human anti-chimaeric antibody (HACA), which suggests that the success of rituximab retreatment would not be limited by a human anti-mouse antibody (HAMA)/HACA response). In addition, the lack of important short- or long-term toxicity suggests that the use of this novel agent does not preclude the subsequent successful use of traditional chemotherapy.

Finally, antibodies such as rituximab can be used with other agents with different mechanisms of action to give enhanced therapeutic benefits. In a recent phase II combination trial,

IDEC-C2B8 demonstrated markedly enhanced responses in patients treated with antibody in combination with conventional chemotherapy [16]. The use of rituximab in combination with radioimmunotherapy [17,18] is also being investigated in a phase II trial. Our studies show optimistically that engineered depleting monoclonal antibodies can be of considerable value in the treatment of carefully selected disease indications through targeted cytotoxic mechanisms *in vivo*.

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FcγRIIIa and FcγRIIa polymorphisms do not predict response to rituximab in B-cell chronic lymphocytic leukemia

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In follicular lymphoma (FL), genomic polymorphisms corresponding to the expression of valine (V) or phenylalanine (F) at amino acid 158 of FcγRIIIa alter the binding affinity of immunoglobulin G1 (IgG1) to the receptor and have been associated with varied responses to rituximab. We examined FcγRIIIa polymorphisms of 30 CLL patients with the phenotypes V/V (n = 6), V/F (n = 12), and F/F (n = 12)

treated with thrice-weekly rituximab (375 mg/m²) for 4 weeks to correlate polymorphism type with infusion toxicity and response. Infusion toxicity (grade 3 or greater or hypoxia/hypotension requiring transient cessation of therapy) was observed equally among the groups (V/V, 50%; V/F, 33%; F/F, 41.6%; *P* = .78). The response to rituximab was also similar among the different polymorphism pheno-

types (V/V, 33%; V/F, 41.6%; F/F, 50%). These data suggest that FcγRIIIa polymorphisms are not predictive of response in CLL and that, unlike the case with FL, mechanisms of tumor clearance other than antibody-dependent cellular cytotoxicity may be more important. (Blood. 2004; 103:1472-1474)

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Introduction

Rituximab is a chimeric monoclonal antibody directed against CD20, an antigen found most B-cell malignancies, including non-Hodgkin follicular lymphoma (FL) and chronic lymphocytic leukemia (CLL). As a single agent, rituximab induces objective responses in more than 50% of FL and CLL patients with minimal toxicity because of its B-cell selectivity.¹⁻⁴ These favorable results have led to considerable interest in combining rituximab with other agents in FL and CLL. However, understanding how rituximab mediates its in vivo biologic effects in these diseases is likely to enhance the effective implementation of combination therapy.

Multiple mechanisms have been proposed for the activity of rituximab, including antibody-dependent cellular cytotoxicity (ADCC),^{5,6} complement-dependent cytotoxicity (CDC),^{6,7} and a direct proapoptotic effect.^{8,9} Although F(ab')₂ rituximab homodimers were shown to be effective in inducing apoptosis of B-cell lymphoma cell lines in vitro,¹⁰ other works have recently established that ADCC is important as a predominant mechanism of lymphoma cell clearance and that Fcγ receptors (FcγRs) are critical for the in vivo actions of rituximab in non-Hodgkin lymphoma (NHL).¹¹ In a xenograft model of human lymphoma, knocking out the FcγR loci in mice showed a complete abrogation of response to rituximab.¹¹ In contrast, knocking out the inhibitory FcγRIIb in mice resulted in enhanced response to rituximab in the same xenograft model.¹¹ In addition, the recent demonstration in NHL patients that the response to rituximab is dependent on specific FcγRIIIa polymorphisms supports the importance of ADCC in the in vivo actions of rituximab.¹² The activating FcγR on natural killer (NK) cells and monocytes (FcγRIIIa) and on

monocytes (FcγRIIa) mediates ADCC. Genomic polymorphism corresponding to phenotype expression of valine (V) or phenylalanine (F) at amino acid 158 on the FcγRIIIa greatly influences the affinity of IgG1 to the Fcγ receptor. Studies have demonstrated the stronger binding of antibody to homozygous FcγRIIIa-158V NK cells than to homozygous FcγRIIIa-158P or heterozygous NK cells.^{13,14} Similarly, polymorphism related to the expression of histidine (H) or arginine (R) at amino acid 131 in FcγRIIa affects the binding affinity of IgG1.¹⁵ Forty-nine patients with previously untreated FL, patients homozygous for FcγRIIIa-158V (V/V), had a significantly higher (100%) clinical response rate than those with heterozygous (V/F) or homozygous phenylalanine (F/F) genotype (67%).¹² Furthermore, polymerase chain reaction (PCR) detection of *bcl-2* rearranged cells was significantly lower in the homozygous V/V group. In contrast, the FcγRIIa-131 polymorphism was not associated with response to rituximab.

It is possible, however, that the relative importance of ADCC as a mechanism for the activity of rituximab may differ in B-cell malignancies other than NHL. CLL differs significantly from FL from an immunophenotypic^{15,16} and a genomic¹⁷ standpoint, with most tumor cells having dim CD20 antigen expression. Studies have demonstrated that CLL cells are often resistant to complement-mediated cytotoxicity^{6,18} and that, in responding patients receiving rituximab,¹⁹ caspase-dependent apoptosis may be the predominant mechanism by which tumor cell elimination occurs. Although the effector-to-tumor cell ratio and the T_H2 cytokine profile in CLL do not favor effective ADCC, the importance of FcγRIIIa has not been explored in CLL. Herein, we examine the importance of FcγRIIIa

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Table 1. Clinical features of CLL patients by FcγRIIIa polymorphism type

| | FcγR V/V, n = 6 | FcγR V/F, n = 12 | FcγR F/F, n = 12 | P |
|--|------------------|------------------|------------------|------|
| Median age, y (range) | 69 (54-79) | 56 (50-74) | 64 (54-79) | .14 |
| No. male (%) | 4 (66) | 7 (58) | 12 (100) | .04 |
| Median WBC count, $\times 10^9/L$ (range) | 59.1 (8.1-175.3) | 64.7 (2.5-152.2) | 72.6 (4.1-173.4) | .80 |
| No. stage III/IV (%) | 5 (83) | 10 (83) | 6 (50) | .32 |
| Mean prior regimens (range) | 3 (1-6) | 2 (0-6) | 2 (0-4) | .39 |
| No. prior nucleoside analog treatments (%) | 5 (83) | 5 (42) | 7 (58) | .24 |
| No. with lymphadenopathy (%) | 5 (83) | 11 (92) | 12 (100) | .39 |
| No. with infusion toxicity (%) | 3 (50) | 4 (33) | 5 (42) | .78 |
| No. partial responses | 2 | 5 | 6 | .70 |
| (95%) CI | 33 (4-78) | 42 (15-72) | 50 (21-78) | > .2 |

WBC indicates white blood cell.

and FcγRIIIa polymorphisms to response and toxicity observed with rituximab treatment in CLL.

Patients, materials, and methods

Patient samples and cell processing

Patients were enrolled in this previously reported, institutional review board (Walter Reed Army Medical Center, Johns Hopkins Oncology Center, and The Ohio State University)-approved, multicenter trial.⁴ All patients gave written, informed consent before participation. Patients were required to have histologically documented CLL as defined by the modified National Cancer Institute (NCI) criteria²⁰ or to have small lymphocytic lymphoma as defined by the International Working Formulation classification.²¹ Treatment included stepped-up administration of thrice-weekly rituximab for 4 consecutive weeks, as previously described.⁴ Response to rituximab was judged 2 months after therapy according to the modified National Cancer Institute (NCI) criteria.²⁰ Infusion toxicity was defined by the presence of hypoxemia, hypotension, or dyspnea, which required transient discontinuation of the infusion and supportive intervention. Cells were obtained before rituximab treatment, and mononuclear cells were isolated from blood using density-gradient centrifugation (Ficoll-Hypaque Plus; Pharmacia Biotech, Piscataway, NJ). Cells were then viably cryopreserved in 10% dimethyl sulfoxide (DMSO), 40% fetal calf serum, and 50% RPMI medium.

Analysis of FcγRIIIa and FcγRIIa polymorphisms

Pretreatment samples for analyses of V/F 158 FcγRIIIa and FcγRIIa 131 H/R polymorphisms were available for 30 (90%) of 33 patients enrolled in this trial. DNA was extracted using the QIAamp kit according to the manufacturer's instructions (Qiagen, Valencia, CA). Assessment of the of FcγRIIIa and FcγRIIa polymorphisms was performed as previously described.¹² All samples were analyzed in duplicate, with identical results.

Statistical analysis

Pretreatment clinical features, observed toxicity, and response to therapy among the polymorphism groups were compared using χ^2 analysis for categorical data and the nonparametric Kruskal-Wallis test for continuous data. A *P* value of .05 was considered statistically significant.

Results

Pretreatment features of the 30 patients separated according to FcγRIIIa polymorphism group are shown in Table 1. Six (20%) and 12 (40%) patients were homozygous for the V/V 158 FcγRIIIa and the F/F 158-FcγRIIIa polymorphisms, respectively. Although more male patients were homozygous for FcγRIIIa F/F, there was no other significant difference in the pretreatment clinical characteristics among the groups. Of the 30 patients included, 12 patients experienced infusion toxicity that required the temporary cessation of rituximab therapy and intervention. The incidence of infusion toxicity was similar (50%, 33%, and 42%; *P* = .78) among the 3 different 158-FcγRIIIa polymorphism groups. Similarly, among the 13 patients who achieved partial response to thrice-weekly rituximab therapy, there was no significant difference in response rate between the V/V, V/F, and F/F 158 FcγRIIIa polymorphism groups, with responses seen in 33% (95% confidence interval [CI], 4%-78%), 42% (95% CI, 15%-72%), and 50% (95% CI, 21%-79%), respectively (*P* = .70). Although the result is limited by the small number of cases studied within each subgroup, resulting in wide confidence intervals for the estimate of response to rituximab, the directional trend in our result was opposite what might have been expected based on the increased affinity of the V/V phenotype FcγRIIIa for the antibody. Table 2 shows the clinical baseline characteristics of the same 30

Table 2. Clinical features of CLL patients by FcγRIIIa polymorphism type

| | FcγR H/H, n = 6 | FcγR H/R, n = 17 | FcγR R/R, n = 7 | P |
|--|------------------|------------------|------------------|-----|
| Median age, y (range) | 68 (51-80) | 57 (50-79) | 74 (64-79) | .03 |
| No. male (%) | 5 (83) | 11 (65) | 7 (100) | .16 |
| Median WBC count, $\times 10^9/L$ (range) | 39.3 (3.4-154.8) | 84.5 (4.8-175.3) | 44.8 (2.5-173.4) | .59 |
| No. stage III/IV (%) | 3 (50) | 13 (77) | 6 (86) | .32 |
| Mean prior regimens (range) | 3 (1-6) | 2 (0-6) | 2 (0-4) | .67 |
| No. prior nucleoside analog treatments (%) | 3 (50) | 10 (59) | 4 (57) | .93 |
| No. with lymphadenopathy (%) | 5 (83) | 16 (94) | 7 (100) | .48 |
| No. infusion toxicity (%) | 2 (33) | 6 (35) | 4 (57) | .57 |
| No. partial responses (95% CI) | 4.67 (22-96) | 5.29 (10-56) | 4.57 (18-90) | .70 |

patients analyzed using the 131-FcγRIIIa polymorphism phenotype: Similar to the results observed for the 158-FcγRIIIa polymorphism, there were no significant differences in infusion toxicity or response rate among the 3 groups.

Discussion

Our findings are in contrast to the FL results reported by Cartron et al,¹² which showed that the high-affinity 158-FcγRIIIa V/V polymorphism was associated with the highest response rate in patients treated with rituximab.¹² Although the numbers of CLL patients in our series was smaller, the subset of patients with the high-affinity polymorphism had the lowest response rate. Several explanations for these findings are possible, including a different mechanism of action of rituximab in CLL compared with lymphoma or a varied patient population among the patient groups analyzed. In FL, preclinical animal studies,¹¹ the FcγRIIIa polymorphism studies of Cartron et al,¹² and higher response rates observed in studies that combine rituximab with agents that enhance ADCC²² all support ADCC as an important mechanism for the activity of rituximab. In contrast, however, our results suggest that ADCC may be a less important mechanism in CLL. The reduced expression of CD20 on the neoplastic cells of CLL compared with FL, or the presence of higher levels of soluble CD20 in CLL, may significantly reduce the efficiency of rituximab in mediating ADCC

in spite of the higher affinity of FcγRIIIa and FcγRIIIa and may contribute to the lack of observed effect of FcγR polymorphisms on response. In addition, it is possible that ADCC is the more important mechanism for clearance of neoplastic B cells in lymph nodes, predominantly involved in FL, compared with blood and bone marrow, which are more commonly involved in CLL. Although we have no direct data to support or refute these possibilities, a plausible alternative explanation is that rituximab acts by different mechanisms in CLL. We previously demonstrated in vivo activation of the intrinsic pathway of apoptosis in CLL patients responding to rituximab.¹⁹ In addition, several cellular and genetic factors that inhibit apoptosis in CLL also diminish response to rituximab. The role of CDC is also less likely because CLL cells often dimly express CD20 and overexpress CD55 and CD59, which prevents CDC.^{6,18} Further diminishing the major role of CDC in CLL is the lack of correlation with response to CD55 and CD59, shown in another study.²³ Indeed, this study, combined with the results of other studies in CLL in which cellular²³ and genetic²⁴ features that disrupt apoptosis are associated with diminished response, provides support for the hypothesis that signaling and apoptosis may contribute more to the therapeutic efficacy of rituximab in CLL than ADCC or CDC. These results provide justification for the emphasis on disease-specific studies to determine the mechanisms of how specific monoclonal antibody therapies work in vivo.

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CD20 levels determine the in vitro susceptibility to rituximab and complement of B-cell chronic lymphocytic leukemia: further regulation by CD55 and CD59

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Complement-dependent cytotoxicity is thought to be an important mechanism of action of the anti-CD20 monoclonal antibody rituximab. This study investigates the sensitivity of freshly isolated cells obtained from 33 patients with B-cell chronic lymphocytic leukemia (B-CLL), 5 patients with prolymphocytic leukemia (PLL), and 6 patients with mantle cell lymphoma (MCL) to be lysed by rituximab and complement in vitro. The results showed that in B-CLL and PLL, the levels of CD20, measured by standard immunofluorescence or using calibrated beads, correlated linearly with the lytic response (coefficient greater than or equal to 0.9; $P < .0001$). Furthermore, the correlation

remained highly significant when the 6 patients with MCL were included in the analysis (coefficient 0.91; $P < .0001$), which suggests that CD20 levels primarily determine lysis regardless of diagnostic group. The role of the complement inhibitors CD46, CD55, and CD59 was also investigated. All B-CLL and PLL cells expressed these molecules, but at different levels. CD46 was relatively weak on all samples (mean fluorescence intensity less than 100), whereas CD55 and CD59 showed variability of expression (mean fluorescence intensity 20-1200 and 20-250, respectively). Although CD55 and CD59 levels did not permit prediction of complement susceptibility, the functional

block of these inhibitors demonstrated that they play an important role in regulating complement-dependent cytotoxicity. Thus, lysis of poorly responding B-CLL samples was increased 5- to 6-fold after blocking both CD55 and CD59, whereas that of high responders was essentially complete in the presence of a single blocking antibody. These data demonstrate that CD20, CD55, and CD59 are important factors determining the in vitro response to rituximab and complement and indicate potential strategies to improve the clinical response to this biologic therapy. (Blood. 2001;98:3383-3389)

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Introduction

The use of therapeutic monoclonal antibodies (MAbs) for the treatment of cancer has become a promising approach over the last few years, as exemplified by the success of the anti-CD20 chimeric MAb rituximab, used for the treatment of B-cell non-Hodgkin lymphoma (B-NHL).¹⁻⁴ Other promising MAbs are emerging, such as Campath-1H (anti-CD52) for the treatment of B-cell chronic lymphocytic leukemia (B-CLL),⁵ anti-CD33 for acute myelocytic leukemia,⁶ anti-p185^{HER2/neu} for breast cancer,⁷ and some others.⁸ The unconjugated forms of these antibodies are thought to act in vivo mostly through activation of antibody-dependent cell-mediated cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC).⁹⁻¹² although direct growth inhibition and/or induction of apoptosis may also take place.^{11,13-15} The relative contributions of these different mechanisms of action are still a matter of debate. The available evidence suggests that activation of ADCC and CDC by rituximab is crucial for in vivo response because an IgG4 version of the antibody does not deplete normal B cells in primates.⁹ Furthermore, complement consumption has been observed in vivo after rituximab administration.¹⁶ We and others have shown previously that follicular lymphoma (FL) cells in vitro can be lysed effectively by rituximab and human complement, although

a high degree of heterogeneity in the response was observed in different cell lines.^{11,12} Furthermore, we have shown in 4 FL cell lines that the levels of the complement inhibitors CD55 and CD59 are at least in part responsible for this heterogeneity.¹¹ Thus, these data using FL cells suggested that complement-mediated lysis is likely to be an important mechanism of action of rituximab in vivo. Furthermore, the data suggested that this mechanism could be the basis for the heterogeneity of the response of different patients to rituximab in vivo.

Rituximab was originally approved for the treatment of low-grade B-NHL, in particular FL, but several trials are in progress testing its activity in mantle cell lymphoma (MCL), hairy cell leukemia (HCL), and chronic lymphocytic leukemia (CLL).¹⁶⁻¹⁹ It is therefore of particular interest to determine the capacity of freshly isolated B cells obtained from patients with different types of leukemias or lymphomas to be lysed by rituximab and complement and to determine the relative roles of CD20 expression levels as well as CD55 and CD59 complement inhibitors in these cells. In the longer term, these studies should help answer the question whether the in vitro response of leukemic cells to rituximab can be predictive of in vivo response and whether easily measurable

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parameters, such as CD20, CD55, and CD59 expression levels, may predict such a response. Such studies may permit us in the future to select patients who will benefit from rituximab therapy and to design new therapeutic protocols, for example blocking of complement inhibitors, to increase significantly rituximab activity *in vivo*.

Materials and methods

Cells

Heparinized peripheral blood was obtained after informed consent from patients with B-CLL, prolymphocytic leukemia (PLL), and MCL with significant circulating disease (at least 50% of neoplastic cells in the mononuclear cell fraction). Except for some B-CLL, all patients' samples were taken at diagnosis. All patients were diagnosed by routine immunophenotypic, morphologic, and clinical criteria. In all cases, double staining with CD19 and sIgk or sIgA was performed, allowing us to establish monoclonality and to determine the percentage of neoplastic versus normal B cells present in the sample. In addition, all patients with MCL were checked for the BCL1 translocation by standard polymerase chain reaction analysis. The cells were separated on a Ficoll Hypaque gradient (Seromed, Berlin, Germany). As controls, mononuclear cell fractions from healthy volunteers were also obtained. In some cases, B cells were purified using the B Cell Isolation Kit, MACS LS separation columns, and a MidiMACS magnetic cell separator (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. Peripheral blood T lymphocytes were purified by Ficoll-Hypaque gradient centrifugation and rosetting with aminoethylisothiocyanate-treated sheep red blood cells using standard procedures. The resulting lymphocytes were 85% CD3⁺. DHL-4 cells have been described previously.¹¹

Immunofluorescence

Blood samples from healthy individuals or neoplastic peripheral blood mononuclear cells (PBMCs) were stained with fluorescein isothiocyanate (FITC)-conjugated anti-CD20 antibody (BD Biosciences, San Diego, CA) or phycoerythrin (PE)-conjugated anti-CD20 (BD Biosciences) together with FITC-conjugated anti-CD46 (BD Biosciences), anti-CD55, or anti-CD59 antibody (Caltag Laboratories, Burlingame, CA) or fluorochrome-labeled control antibodies. Samples were analyzed in single or double immunofluorescence on a FACScan instrument or on a FACS Calibur (BD Biosciences). To compare staining intensities for different patients on different days, the negative control curves for all patients were set between the first and 10th channel of fluorescence, and the gate for positive cells was set at or near channel 10. Furthermore, for a number of samples, the absolute number of CD20 molecules was measured using calibrated Quantibrite beads (BD Biosciences) and PE-labeled anti-CD20, following the manufacturer's instructions.

For complement deposition, the anti-C9 MA bE11 and an anti-C3 goat polyclonal antiserum were used (kindly provided by Dr F. Tedesco, University of Trieste, Italy), together with the appropriate FITC-conjugated secondary antibodies (Jackson Immunoresearch Laboratories, West Grove, PA; and Sigma, St Louis, MO, respectively).

Complement-mediated lysis

Complement cytotoxicity assays were performed essentially as described,^{11,20} with some modifications. Briefly, 50 000 cells/well were placed in 60 μ L in triplicate in 96-well plates in the presence of 10 μ g/mL rituximab and/or 10 μ g/mL functionally blocking anti-CD55 (BRIC216; International Blood Group Reference Laboratory (IBGRL), Bristol, United Kingdom) or anti-CD59 (BRIC229; IBGRL) antibodies and in the presence or absence of pooled human serum to a final concentration of 25%. The cells were incubated for 4 hours at 37°C, then diluted with medium to 270 μ L, and 1/10 volume of Alamar blue solution was added (Biosource International, Camarillo, CA). Incubation was performed overnight at 37°C, and the plates were read in a fluorimeter (Cytofluor 2300; Millipore, Bedford, MA) with excitation at 530 nm and emission at 590 nm.²⁰ In all

cases, the effect of rituximab alone in the absence of human serum was determined. The samples with human serum alone were used as negative controls to normalize for the quenching of fluorescence by serum proteins and for the presence of dead cells in the samples in the absence of any treatment. According to the manufacturer's instructions (Biosource), 0.25% Triton-X100 (Sigma) was added to the wells used to set up the background fluorescence (all cells lysed). To confirm the validity of the data, we performed the same assay on at least 10 samples with 10⁶ cells/mL and analyzed the percentage of dead cells using acridine orange staining and fluorescence-activated cell sorter (FACS) analysis, as described previously.¹¹ Furthermore, the linearity of the Alamar blue assay was verified using serial dilutions of cells. Relative lysis was obtained using the following formula: percentage lysed cells/percentage CD20⁺ cells \times 100. In some experiments, cells were stained with 0.5 μ g/mL propidium iodide (PI; Sigma) to detect dead cells by FACS analysis.

Statistical analysis

Statistical analysis was performed using the Stat4.5 program on a Macintosh power computer. The Pearson correlation coefficient was obtained for all patients, either individually or grouped together. In the CD55 and CD59 functional assays, 2-way analysis of variance (ANOVA) was performed. To analyze the effects of blocking antibodies against their controls, we performed a *t* test for paired data. One-way ANOVA was performed to evaluate the differences between the different antibody treatments.

Results

Specificity of CDC assays

Because we planned to analyze the complement-mediated lysis (CDC) of a large panel of freshly isolated mononuclear cell samples that contained different percentages of CD20⁺ cells, we first investigated the specificity of lysis in heterogeneous samples. The DHL-4 cell line, which expresses high levels of CD20 and is lysed extremely rapidly (within 15 minutes) after the addition of rituximab and complement,¹¹ was mixed with freshly isolated purified peripheral blood T lymphocytes from healthy donors. Lysis was then performed on this mixed population using rituximab and human complement at standard concentrations. As control, the same cell mix was incubated with human serum alone. Because

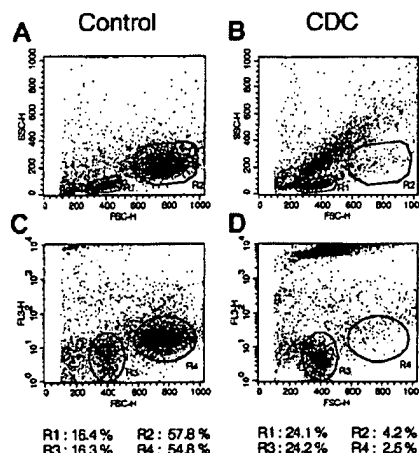


Figure 1. Specificity of rituximab and complement-mediated lysis. Peripheral blood T lymphocytes were mixed with DHL-4 B cells and incubated for 3 hours at 37°C with 25% human serum alone (A,C; control) or in the presence of 10 μ g/mL rituximab (B,D; CDC). Cells were then washed, stained with propidium iodide, and analyzed on the FACS. (A,B) Results of scatter analysis. (C,D) Results of dot plots of the red fluorescence. The percentages of cells in both the control and CDC panels are shown at the bottom. The results are representative of 2 separate experiments.

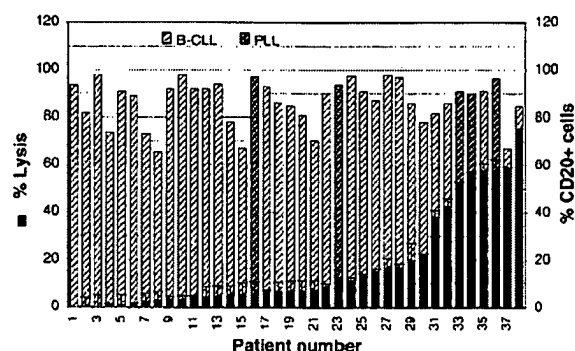


Figure 2. Complement-mediated lysis of B-CLL cells. CDC assays were performed on the PBMCs isolated from 33 patients with B-CLL and 5 with PLL. Percentage lysis in the presence of 10 μ g/mL rituximab and 25% human serum is shown (black bars). The percentage of cells expressing CD20 was also evaluated by standard direct immunofluorescence (hatched bars). The PLL samples are identified by darker hatched bars.

phenotypic analysis of lysed cells was not possible as a result of high nonspecific staining of dead cells, cell death was analyzed using FACS scatter analysis (Figure 1A-B) as well as PI staining (Figure 1C-D). The 2 cell populations (DHL-4 and T lymphocytes) could be easily distinguished by scatter analysis (gate R1 for lymphocytes and gate R2 for DHL-4; Figure 1A). Scatter analysis was confirmed by staining with anti-CD3 and anti-CD19 antibodies, which showed that R1 cells were 85% CD3⁺ and R2 cells were 90% CD19⁺ (data not shown). Addition of rituximab and complement led to nearly complete lysis of the DHL-4 cells, as shown by a change in scatter of R2 cells, which passed from 54.8% before lysis to 4.2% of the total cell population (Figure 1B). Rituximab and complement did not, however, affect T cells in gate R1, which increased from 16.4% to 24.1% (Figure 1B). As expected, R1 cells remained 87% CD3⁺ after lysis (data not shown). A small increase in the percentage of R1 cells was observed in 2 different experiments and was probably due to a decrease in total cell number resulting from complete rupture in small fragments of some DHL-4 cells by complement. Red fluorescence analysis confirmed that a change in scatter was accompanied by PI uptake above control levels in DHL-4 cells (Figure 1C-D; gate R4), but not in T lymphocytes (gate R3). This demonstrates the extreme specificity of lysis for CD20⁺ cells, even in the presence of strong and rapid complement activation.

Complement-mediated lysis of B-CLL and PLL is determined by CD20 levels

We have shown previously that complement-mediated lysis of FL cell lines is highly heterogeneous and is regulated by the CD55 and CD59 complement inhibitors. To determine whether rituximab and complement can efficiently kill other subtypes of leukemias or lymphomas, we analyzed a large panel of freshly isolated B-CLL and PLL samples. These pathologic entities were grouped in the analysis because the latter represents a clinical and morphologic variant of B-CLL, the 2 show an overlapping phenotype, and the results obtained with the 2 groups of patients were found to be overlapping (see below). Mononuclear cells were isolated from the peripheral blood of 33 patients with B-CLL and 5 with PLL. The percentage of CD20⁺ cells for each of these cell populations was determined by immunofluorescence and ranged from 65% to 98%, with a mean of 82% (Figure 2, hatched bars). The 5 PLL samples are identified with darker hatched bars. Double staining with CD19 and $\text{slg}\kappa$ or $\text{slg}\lambda$ showed that normal B cells were present at less

than 3% in all cases, assuming a normal κ to λ ratio of 2:1 (data not shown).

The patients' cells were then incubated in the presence of rituximab and human complement for 4 hours, and cell death was determined using the Alamar blue assay.²⁰ The samples have been ordered according to their susceptibility to CDC (Figure 2). As shown in Figure 2 (black bars), most samples were lysed poorly with rituximab and complement. Indeed, 22 of 38 samples (58%) showed lysis of less than 10%, and another 8 cases (21%) were below 25%. Only 8 samples showed lysis of greater than 30%, which in fact corresponded to lysis of 50% or more of CD20⁺ cells (Figure 2). Among cases with efficient lysis, 3 were PLL and 5 were B-CLL.

The effect of rituximab alone in the absence of human serum was also analyzed in 21 cases of B-CLL and PLL, including 7 high responders. The measured cell death ranged from 0% to 6% in different samples, with a mean of 3.1%, suggesting little effect of rituximab alone under these experimental conditions (data not shown).

Because B-CLL cells are generally known to express relatively low levels of CD20^{21,22} and also show little susceptibility to rituximab and complement (Figure 2), we wondered whether the extent of lysis was determined by the levels of expression of CD20 itself. Thus, the mean fluorescence intensity (MFI) for CD20 for each sample was plotted against the relative lysis (ie, the percentage lysis normalized for the total percentage of CD20⁺ cells in the sample). As shown in Figure 3A, the relative lysis correlated highly significantly with the MFI of CD20 (correlation coefficient 0.92; $P < .0001$). Interestingly, the correlation was high even though standard FACS analysis was performed using 2 different FACS apparatus and FACS operators (who analyzed 18 and 20 samples, respectively). Furthermore, the correlation was significant for the B-CLL and PLL samples also when analyzed separately (coefficients 0.92 and 0.87, respectively), although significance was relatively low for PLL ($P = .05$) because only 5 samples could be analyzed in this case. A high significance ($P < .0001$) remained, however, for the 33 B-CLL cases when analyzed separately from

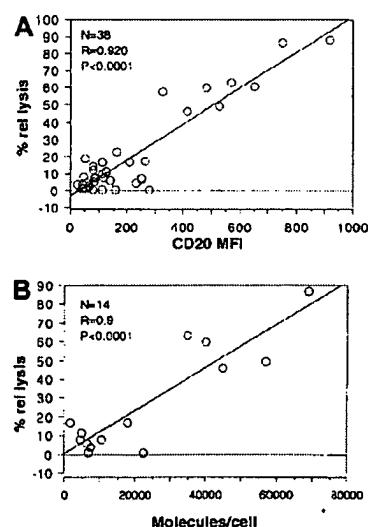


Figure 3. Complement-mediated lysis correlates with the intensity of CD20 expression. (A) The mean fluorescence intensity of CD20 was plotted against the relative lysis obtained in the presence of rituximab and complement for each of the 33 B-CLL and 5 PLL patients shown in Figure 2. (B) The number of molecules per cell measured for 14 B-CLL/PLL patients was plotted against the percentage relative lysis for the same patients. The results of the statistical analysis are shown in each plot.

the PLL cases. These results justify the grouping of B-CLL and PLL in the analysis.

To verify that standard immunofluorescence analysis gives results that correspond to the number of molecules actually expressed, we analyzed 14 B-CLL cases that covered the whole spectrum of CD20 intensities for CD20 expression with the use of calibrated beads. As shown in Figure 3B, CD20 expression ranged from 1500 to 70 000 molecules per cell. Furthermore, rituximab and complement-mediated lysis also significantly correlated with the number of CD20 molecules per cell (coefficient 0.9; $P < .0001$), even though the number of samples analyzed was relatively small.

These data show that in CLL/PLL samples that express variable levels of CD20, rituximab- and complement-mediated lysis depends primarily on the levels of expression of CD20 itself.

Expression and role of the CD55 and CD59 complement inhibitors in B-CLL and PLL

Because surface-associated complement inhibitors have been shown previously to regulate CDC in FL cells,¹¹ we also investigated in B-CLL and PLL the patterns of expression of the CD46, CD55, and CD59 complement inhibitors on CD20⁺ cells by double immunofluorescence analysis. All B-CLL and PLL cells were 95% to 100% positive for all 3 complement inhibitors (data not shown). However, the intensity of expression varied among samples. In particular, CD55 was expressed at different levels in different samples, with an MFI ranging from 20 to 1150 in 37 patients analyzed (Figure 4A, closed circles for B-CLL and squares for PLL). CD59 showed some variability in expression, although less than that of CD55; the CD59 MFI ranged from 20 to 224 in 37 samples tested (Figure 4A). Finally, CD46 expression was quite constant, with an MFI ranging from 17 to 68 in 29 patients analyzed (Figure 4A). The 5 PLL samples analyzed did not differ from the B-CLL samples for complement inhibitor expression, again justifying their inclusion within the same group.

To determine complement inhibitor expression in normal B lymphocytes, we also analyzed peripheral blood B cells from 4 healthy volunteers by double immunofluorescence. As shown in

Figure 4A (open circles), CD55 and CD59 were expressed at similar levels in all 4 samples. Interestingly, CD55 was low (MFI 26-54) in all 4 samples, whereas CD59 was higher in all cases. Indeed, the ratio of CD55 to CD59 for normal B cells ranged from 0.2 to 0.3 (mean, 0.23), whereas it was 1 to 15 (mean, 4.4) for all B-CLL and PLL patients. Thus, B-CLL and PLL have complement inhibitor expression skewed toward higher CD55 relative to CD59.

The relatively high and variable expression of CD55 and CD59 in patients led us to investigate whether there was any inverse correlation between complement lysis and CD55 or CD59 expression. As shown in Figure 4B and C, no significant correlation could be found because the coefficient was close to 0 in all cases (37 cases analyzed). Again, performing the analysis for B-CLL or PLL separately did not change the result (data not shown). Thus, the levels of expression of CD55 or CD59, which are expressed on all B-CLL cells, are not predictive of lysis in these pathologies.

The functional role of CD55 and CD59 can be investigated more directly by performing complement lysis assays in the presence of antibodies that functionally block these molecules but are unable by themselves to activate complement²³ (also, data not shown). This assay was performed on all 38 B-CLL and PLL samples. Figure 5A shows the results obtained with 6 representative B-CLL cases. The results show that anti-CD55 or anti-CD59 and, more effectively, both antibodies together increased the lytic response, although to a variable degree in different patients. In some patients showing basal CDC of less than 10% (eg, cases 5 and 10), lysis was increased 2- to 3-fold with a single blocking antibody and up to 10-fold with both anti-CD55 and anti-CD59. In cases showing high basal CDC (more than 50%; eg, cases 35 and 38), lysis was complete by adding either CD55 or CD59 blocking antibodies singly. Figure 5B shows the mean percentage lysis with or without blocking the CD55 and/or CD59 inhibitors calculated from all samples. The data demonstrate that anti-CD55 or anti-CD59 increased the mean lysis by an average of 2-fold, whereas the combination of antibodies augmented it by nearly 3-fold (Figure 5B). These increases are statistically significant ($P < .0001$). The effect of the combined relative to single antibodies is also statistically significant ($P < .0001$). Because the basal CDC response (rituximab plus complement alone) was highly variable among different samples (ranging from 0% to 75%), the effect of blocking CD55 and/or CD59 could be different in patients who show a low or high basal CDC. The mean levels of lysis were therefore split in Figure 5C between low responders (30 samples with basal lysis less than 25%) and high responders (8 samples with basal lysis greater than 30%). The results show that the effect of the blocking antibodies was proportionally greater in low responders, with a 2.8-, 3.6-, and 5.5-fold increase for CD55, CD59, and both antibodies together, respectively. In high responders, lysis was nearly complete after adding only a single blocking antibody because the mean percentage of CD20⁺ cells for this group was 87%.

We conclude that in B-CLL and PLL, all of which express detectable levels of CD55 and CD59, both complement inhibitors effectively block complement activation. However, CD55 and CD59 levels of expression do not allow prediction of the extent of lysis.

Complement-mediated lysis of MCLs

Because clinical trials are being conducted to test the activity of rituximab in MCL,^{17,24,25} we extended our analysis of CDC to a smaller number of fresh MCL samples. Because the percentage of CD20⁺ cells in the peripheral blood of these patients is in some cases relatively low (45% to 50%), we first verified that the Alamar

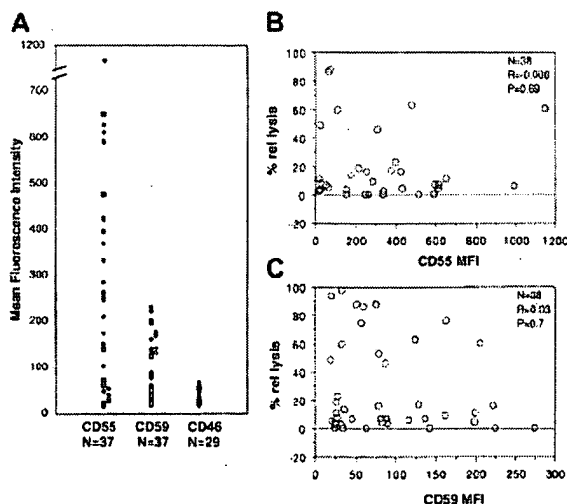


Figure 4. Analysis of CD55 and CD59 expression and function in B-CLL/PLL. (A) PBMCs from 33 patients with B-CLL (closed circles) and 4 patients with PLL (closed squares) as well as 4 healthy volunteers (open circles) were double stained with anti-CD20-PE and anti-CD55-FITC, anti-CD59-FITC, or anti-CD46-FITC antibodies and analyzed on the FACS. (B) The CD55 MFI was plotted against the percentage relative lysis for the same patients. (C) The CD59 MFI was plotted against the relative lysis. The results of the statistical analysis are shown in each plot.

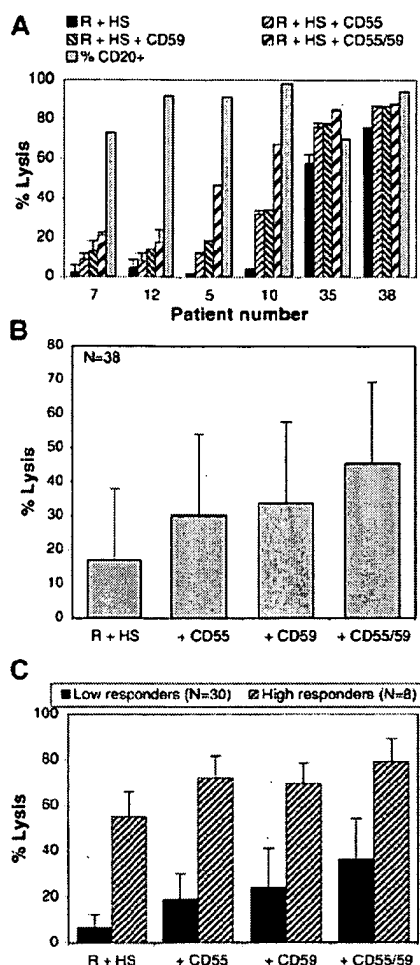


Figure 5. CD55 and CD59 block rituximab and complement-mediated lysis. PBMCs from 33 patients with B-CLL and 5 patients with PLL were lysed with rituximab and 25% human serum in the presence or absence of blocking anti-CD55 and/or anti-CD59 antibodies (10 μ g/mL). Lysis was measured after 4 hours with the Alamar blue assay. (A) The mean and SDs obtained with 6 different representative patients are shown. (B) The mean lysis and SDs for all 38 patients are shown. (C) The mean lysis of the 30 low responders (basal lysis less than 25%; black bars) and of the 8 high responders (basal lysis greater than 30%; hatched bars) and SDs are shown.

blue CDC assay was sufficiently specific in such samples. CDC was performed on a sample containing 50% CD20⁺ cells, and the same sample was then subjected to negative selection to purify B cells (to 98% purity), and CDC was performed on these cells in parallel. In addition, CDC was performed in the presence or absence of the anti-CD55 or anti-CD59 blocking antibodies. As shown in Figure 6, lysis of unpurified and purified samples with rituximab and complement was 42% and 96%, which corresponded to lysis of 84% and 98% of CD20⁺ cells, respectively, after adjustment for the percentage of CD20⁺ cells in the sample. Addition of both CD55 and CD59 blocking antibodies increased lysis to 49% for unpurified cells and 98% for purified cells, which corresponded in both cases to greater than 98% of CD20⁺ cells. These data demonstrate that the assay can accurately measure CDC even in samples containing relatively low numbers of CD20⁺ cells. Although the assay could not distinguish between neoplastic and contaminating normal B cells, double immunofluorescence analysis of CD19 and sIg κ/λ showed that contaminating nonnaïve B cells were present at less than 3% in all cases (data not shown). Such a

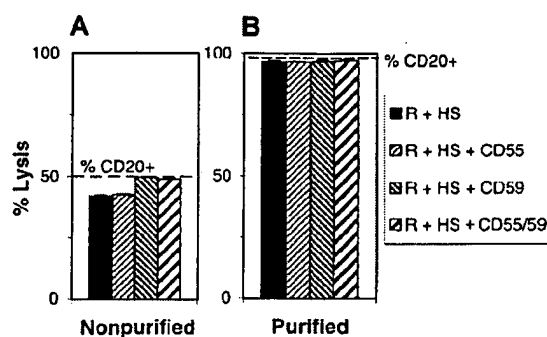


Figure 6. Specificity of lysis of MCL samples. (A) PBMCs from a patient with MCL containing 50% CD20⁺ cells were lysed with rituximab and 25% human complement in the absence (black bars) or presence (hatched bars) of anti-CD55 and/or anti-CD59 antibodies (10 μ g/mL). The percentage of CD20⁺ cells was determined and is indicated by the broken line. (B) The MCL B cells were purified by negative selection, reaching 98% CD20⁺, and lysis was repeated as in (A).

small component in the sample cannot influence significantly the results obtained.

Peripheral blood samples from 6 patients with MCL, which contained 45% to 98% CD20⁺ B cells (mean, 74%), were then analyzed for CDC in the presence or absence of CD55 and/or CD59. As shown in Figure 7 (black bars), lysis with rituximab and complement alone was high in all cases (54% to 98% of CD20⁺ cells). These data are in agreement with those obtained earlier for B-CLL and PLL because MCLs express relatively high levels of CD20 (MFI greater than or equal to 400). Although the MCL patients were too few and too homogeneous to allow an analysis of correlation between CD20 expression and CDC on their own, they were analyzed together with the 33 B-CLL and 5 PLL samples. The data shown in Figure 8 demonstrate that a high correlation was still observed between relative lysis and CD20 expression (coefficient 0.91; $P < .0001$) when all 44 patients were grouped together. These data suggest that CD20 levels can predict sensitivity to rituximab regardless of diagnostic group, at least within the pathologic entities examined (B-CLL, PLL, and MCL).

With regard to CD55 and CD59 function in MCL, Figure 7 shows that blocking either molecule significantly increased lysis of those samples that were not lysed completely with rituximab alone (cases 1, 3, 4, and 5). Anti-CD55 and anti-CD59 were equally effective and in several cases (nos. 1, 4, and 5) one blocking antibody alone was sufficient to induce complete lysis (Figure 7).

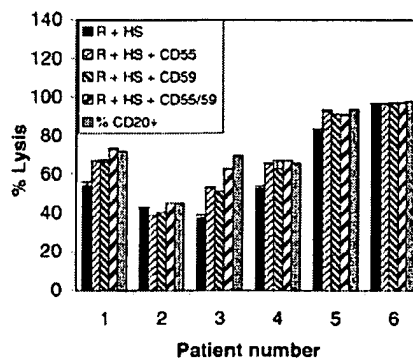


Figure 7. Rituximab and complement-mediated lysis of MCLs. PBMCs from 6 cases of MCL were lysed with rituximab and 25% human serum (black bars) in the presence or absence of blocking anti-CD55 and/or anti-CD59 antibodies (10 μ g/mL; hatched bars). Lysis was measured with the Alamar blue assay. The percentage CD20⁺ cells in each sample is also shown (gray bars).

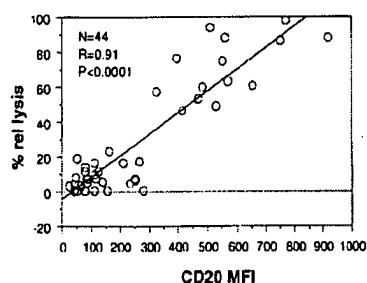


Figure 8. CDC correlates with CD20 expression levels in all patients (CLL, PLL, and MCL). The CD20 MFIs of the 33 B-CLL, 5 PLL, and 6 MCL patients were plotted against the relative lysis obtained with rituximab and complement for the same patients. The results of the statistical analysis are shown.

Complement deposition

To analyze in more detail the complement cascade on fresh neoplastic B cells, we selected 4 cases with sufficient cell numbers that differed in CD20 expression. We then measured the deposition of C3 and C9 complement fragments on the cell surface after exposure to rituximab and complement *in vitro*. As shown in Table 1, the percentage of cells positive for C3 was high in all cases (greater than 80%), showing that C3 deposition occurred on all B cells. However, the intensity of C3 varied significantly in the different samples, and this correlated with CD20 expression. PLL 2 cells, which expressed little CD20 (MFI 80), also showed the lowest level of C3 deposition (MFI 294). As expected, the other cases, which showed CD20 expression ranging from 480 to 530, also had higher C3 deposition (MFI 599-875). The results with C9 were somewhat different in that, in this case, the percentage of cells that became C9 positive correlated approximately with the percentage of cells killed by rituximab, whereas C9 intensity was similar between samples. Although this analysis was performed on few samples, the data show in a direct way that the complement cascade is triggered more efficiently by rituximab in the presence of a higher CD20 density on the cell surface. Furthermore, the data show that in cells that are lysed poorly, the completion of the complement cascade is efficiently counteracted by complement inhibitors, which are likely to include CD55 and CD59.

Discussion

Several lines of evidence suggest that the mechanism of action of rituximab *in vivo* may include complement-mediated cell lysis.^{9,11,12,16} These considerations have led us to investigate the response to rituximab and complement of several subtypes of fresh B-cell leukemias and lymphomas *in vitro*. In addition, we determined whether the levels of expression of CD20 and the complement inhibitors CD55 and CD59 could predict killing by complement *in vitro*. Finally, we analyzed directly the role of the CD55 and CD59 inhibitors using MAbs that functionally block these molecules.

Our data demonstrate that CDC triggered by rituximab is extremely specific to CD20⁺ cells in a mixed population. Analysis of 33 B-CLL and 5 PLL freshly isolated samples showed that they are heterogeneous in their susceptibility to rituximab and complement, with lysis ranging from 0% to 90% of CD20⁺ cells. Analysis of CD20, CD55, and CD59 expression levels by standard immunofluorescence showed unambiguously that lysis correlated closely with CD20 MFI, but not with either CD55 or CD59. The correlation with CD20 expression level was highly significant

statistically, and the same results were obtained when analyzing the individual diagnostic groups either separately or together, further confirming the validity of the results. A number of samples were also analyzed using calibrated fluorescent beads to quantify the number of CD20 molecules per cell. These data showed that CD20 expression in B-CLL/PLL varied from 2000 to 70 000. The data also showed that lysis correlated highly significantly with the number of CD20 molecules per cell, as expected, confirming the validity of the standard immunofluorescence measurements. These results suggest that standard immunofluorescence analysis is sufficient to predict CDC *in vitro* and could be applied to analyze the role of CD20 expression in the *in vivo* response of different patients to rituximab.

The analysis of sensitivity to rituximab and complement was also extended to 6 cases of MCL. All MCLs tested showed relatively high lysis (54% to 98%) and also high CD20 expression levels (MFI greater than or equal to 400). Thus, a highly significant correlation between lysis and CD20 expression levels was observed when analyzing all 44 B-CLL, PLL, and MCL patients together. These data show that the CDC response is primarily determined by the levels of CD20, regardless of diagnostic group, at least within the pathologies examined. In this regard, it is worth noting that 2 HCL cases were also analyzed for CD20 expression and CDC. Both cases showed high CD20 levels (MFI greater than 1000) and also complete lysis in the presence of rituximab and complement, suggesting that the correlation may apply to other B-cell neoplasias as well (data not shown).

As expected from the correlation analysis, CD20 levels determined the extent to which the complement cascade was triggered by surface-bound antibody. Indeed, C3, which reflects the initiation of the cascade, was deposited on all cells, but to a different extent depending on the levels of expression of CD20 itself.³⁶ In contrast, C9 deposition was detected only on cells undergoing lysis, demonstrating that complement inhibitors are present that inhibit completion of the complement cascade.²⁶

Both the expression and functional roles of the CD55, CD59, and CD46 complement inhibitors were investigated. Expression of CD55 was found to be heterogeneous in B-CLL/PLL, varying up to 100-fold in MFI. CD59 expression also showed some variability, with approximately a 10-fold difference in MFI. Interestingly, the mean ratio of CD55 to CD59 was reversed in B-CLL/PLL patients relative to that observed in peripheral blood B cells from 4 healthy donors. Although the number of normal B cells was small, the data suggest that in B-CLL/PLL, complement inhibitor expression is skewed toward a relatively high CD55 expression. Further work will be required to determine whether high CD55 is characteristic also of normal CD5⁺ B cells and is beyond the scope of this article.

Although we found no correlation between levels of CD55 or

Table 1. C3 and C9 complement fragment deposition *in vitro*

| Patient | Percentage C3-positive cells (MFI) | | Percentage C9-positive cells (MFI) | | Lysis (%) |
|---------|------------------------------------|----------|------------------------------------|---------|-----------|
| | R | R + HS | R | R + HS | |
| PLL 2 | 25 (23) | 88 (294) | 1 | 3 (46) | 11 |
| PLL 4 | 2 (108) | 86 (875) | 1 | 43 (21) | 58 |
| CLL 32 | 23 (95) | 81 (599) | 0 | 50 (20) | 42 |
| MCL 1 | 22 (127) | 83 (799) | 0 | 59 (17) | 54 |

PBMCs from the indicated patients were incubated with rituximab in the presence or absence of 25% human serum for 3 hours, washed, and stained with antibodies specific for the indicated complement fragments by indirect immunofluorescence. MFI indicates mean fluorescence intensity; R, rituximab only; R + HS, rituximab and human serum; PLL, prolymphocytic leukemia; CLL, chronic lymphocytic leukemia; MCL, mantle cell lymphoma.

CD59 expression and lysis, direct analysis of the effect of CD55 and CD59 using functionally blocking MABs demonstrated that these are important inhibitors of the complement cascade in neoplastic B cells. Both anti-CD55 and anti-CD59 antibodies were effective to a similar extent, and the effect was most dramatic after adding both blocking antibodies together. The effect was most notable in cells having a low basal response, in which a mean 6-fold increase in lysis could be obtained with both blocking antibodies. On the other hand, in many cases showing relatively high basal lysis, addition of only one blocking antibody was sufficient to lead to complete lysis. This finding may be important in the context of resistance to rituximab in vivo, where increasing rituximab activity may allow elimination of residual resistant cells.⁴ The fact that lysis did not correlate with CD55 or CD59 expression suggests that even low levels of CD55 or CD59 are sufficient to efficiently inhibit the complement cascade, because all patient samples tested expressed the inhibitors, albeit at relatively low levels in some cases. Thus, increasing CD55 and/or CD59 expression above that level does not appear to increase their capacity to inhibit complement.

Although several lines of evidence suggest that CDC is one of the mechanisms of action of rituximab in vivo,^{9,11,12,16} it is likely that other mechanisms operate, such as ADCC and apoptosis. Dissecting the contribution of CD20 or of other factors for each of these mechanisms will be obviously important. Under the experimental conditions used here (4 hours' incubation with rituximab without cross-linking), we were unable to detect significant cell death in the absence of complement. We have therefore no

evidence that significant apoptosis can take place in the different neoplastic B cells examined here. We cannot exclude, however, that cross-linking and/or longer incubation times or other factors could allow induction of apoptosis in these same cells. Such study is beyond the scope of this article.

The variability in the response of fresh leukemic cells to complement in vitro may reflect the heterogeneity in the response of leukemic patients to this drug in vivo. Furthermore, particularly for B-CLL, patients showing stronger lysis in vitro may be those more at risk of developing infusion-related side effects.^{19,27} Indeed, a relatively poor response of B-CLL patients to rituximab has been reported, as well as some cases of life-threatening tumor lysis syndrome,^{16,19,27} which would correlate with the data presented here. Even though it is evident that other factors, such as tumor mass and systemic disease, influence the overall response in vivo,²⁸ we propose that the simple, reproducible, inexpensive, and rapid quantitative assay of CDC on fresh leukemic samples, such as described here, should be a valuable tool to predict the in vivo response of different patients. The need for a relatively small quantity of cells for this assay should allow its applicability also to neoplastic cells isolated from lymph node biopsies. Finally, our data strongly suggest that inhibiting the CD55 and/or CD59 antigens in vivo could markedly improve the biologic activity of rituximab.

Acknowledgments

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Rituximab Infusion Promotes Rapid Complement Depletion and Acute CD20 Loss in Chronic Lymphocytic Leukemia¹

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Complement plays an important role in the immunotherapeutic action of the anti-CD20 mAb rituximab, and therefore we investigated whether complement might be the limiting factor in rituximab therapy. Our *in vitro* studies indicate that at high cell densities, binding of rituximab to human CD20⁺ cells leads to loss of complement activity and consumption of component C2. Infusion of rituximab in chronic lymphocytic leukemia patients also depletes complement; sera of treated patients have reduced capacity to C3b opsonize and kill CD20⁺ cells unless supplemented with normal serum or component C2. Initiation of rituximab infusion in chronic lymphocytic leukemia patients leads to rapid clearance of CD20⁺ cells. However, substantial numbers of B cells, with significantly reduced levels of CD20, return to the bloodstream immediately after rituximab infusion. In addition, a mAb specific for the Fc region of rituximab does not bind to these recirculating cells, suggesting that the rituximab-opsonized cells were temporarily sequestered by the mononuclear phagocytic system, and then released back into the circulation after the rituximab-CD20 complexes were removed by phagocytic cells. Western blots provide additional evidence for this escape mechanism that appears to occur as a consequence of CD20 loss. Treatment paradigms to prevent this escape, such as use of engineered or alternative anti-CD20 mAbs, may allow for more effective immunotherapy of chronic lymphocytic leukemia. *The Journal of Immunology*, 2004, 172: 3280–3288.

The anti-CD20 mAb rituximab (RTX)⁴ was the first mAb approved for single agent therapy for non-Hodgkin's lymphoma (NHL) (1–6). The efficacy of this mAb in indolent or follicular NHL is well documented, and it is also being examined as an immunotherapeutic agent against chronic lymphocytic leukemia (CLL) (7–10). The mechanism of antitumor activity of RTX *in vivo* remains a subject of some debate; preclinical studies, as well as more recent reports of animal models and clinical investigations have provided support for apoptosis (11–14), Fcγ receptor-mediated Ab-dependent cellular cytotoxicity (ADCC) (10, 15–18), and complement-dependent cytotoxicity (CDC) (19–28). In patients selected for treatment based on relatively low circulating lymphocyte counts (<5000/μl), infusion of RTX leads to rapid and prolonged depletion of normal and malignant B cells from the

bloodstream (1, 4). This rapid clearance may be attributed to complement-mediated lysis and/or phagocytosis of B cells via Fcγ receptors on cells of the mononuclear phagocytic system (MPS).

We have reported that binding of RTX to CD20⁺ cells promotes complement activation and covalent deposition of approximately half a million C3b activation fragments (C3b(i)) per cell (26). Our fluorescence microscopy experiments indicate that most deposited C3b(i) is colocalized with bound RTX on B cell lines and on primary CLL cells (26); based on studies in model systems, it is likely the C3b(i) is covalently bound to RTX (29). These findings, along with the observed RTX-mediated clearance of CD20⁺ cells from the bloodstream, raise several important issues.

First, RTX treatment for CLL under conditions of high cell burden might consume so much complement that the ability of RTX to promote CDC could be compromised. Second, the capacity of the MPS to remove IgG-opsonized cells may be exceeded at high cell counts. Third, studies of phagocytosis reported by Griffin et al. (30) suggest that Fcγ receptor-mediated rearrangement and capping of RTX-CD20 complexes on the surface of B cells might lead to removal of the complexes by macrophages instead of whole cell phagocytosis, thus allowing the cells to escape.

We addressed these issues by studying the effects of RTX treatment on complement levels in patients with B cell lymphomas. We evaluated C3b(i) deposition and its colocalization with bound RTX on CD20⁺ cells from RTX-treated CLL patients, taken before, during, and after RTX infusion. Our results indicate RTX infusion promotes complement consumption in CLL, and sera taken from such patients after RTX treatment have reduced capacity to lyse Raji and primary lymphoma cells due to complement depletion. We also observed acute loss of CD20 from B cells present in the circulation immediately after RTX treatment. The details of these findings, and the demonstration that cytotoxic activity can be substantially restored in RTX-rich, C-depleted CLL patient sera by

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⁴ Abbreviations used in this paper: RTX, rituximab; ADCC, Ab-dependent cellular cytotoxicity; AL, Alexa dyes; APbCy, allophycocyanin; CDC, complement-dependent cytotoxicity; CH50, total complement hemolytic activity; CLL, chronic lymphocytic leukemia; MESF, molecules of equivalent soluble fluorochrome; MPS, mononuclear phagocytic system; NHL, non-Hodgkin's lymphoma; NHS, normal human serum; RT, room temperature.

supplementation with normal human serum (NHS) or complement component C2, form the basis for this study.

Materials and Methods

Cell lines, sera, and patients

ARIH77, DB, and Raji cells were obtained from American Type Culture Collection (Manassas, VA) and maintained, as described (26). Blood was obtained with written informed consent from healthy volunteers or patients diagnosed with B cell lymphomas; the University of Virginia (UVA) Institutional Review Board approved all protocols. PBMC were isolated from anticoagulated whole blood by density-gradient centrifugation with Ficoll-Paque^{plus} (Amersham Pharmacia Biotech, Piscataway, NJ) (31). Viability defined by trypan blue exclusion was >95%. Patient and NHS were processed within 1 h after blood collection and stored at -80°C .

Patients with B cell lymphomas received one or more cycles of RTX therapy (375 mg/m^2 , once per week for 4 wk (1, 2, 4)), except for CLL patient 22, who had two infusions of RTX, 5 wk apart. Other than patient 22, patients were treated as outpatients. This factor and the need to restrict the amount of blood taken limited the total number of blood samples available for analysis. Blood samples were collected immediately before and after RTX infusion and in several cases after 30 mg of RTX was infused. Complete blood counts were determined by clinical laboratories at the UVA Hospital. Of 25 patients studied, 6 had CLL, and the results for 4 of these CLL patients (patients 1, 9, 22, and 33) with respect to complement consumption and loss of CD20 are reported in detail. CLL patient 8 had low CD20 levels and a low complement titer before treatment. CLL patient 10 had normal complement levels, but low CD20, and complement was not consumed when this individual was treated with RTX.

Antibodies

IgG1 mAbs 7C12 and 3E7 (specific for C3b/iC3b), IgG2a mAb 1H8 (specific for C3b/C3b/C3dg), and IgG1 mAb HB43 (specific for the Fc region of human IgG) have been described (26, 32). Our findings with the mAbs cited above are generally identified by their specific epitopes, e.g., mAb HB43 was used to detect the human Fc region of RTX, and is referred to as anti-RTX. RTX (IDEC Pharmaceuticals, San Diego, CA) was purchased at the hospital pharmacy. mAbs were labeled with Alexa (Al) dyes (Molecular Probes, Eugene, OR), following the manufacturer's directions. Other mAbs included (label and epitope identified first): PE anti-CD5, 5D7, IgG1 (Caltag Laboratories, Burlingame, CA); PE and allophycocyanin (APhCy) anti-CD19, SJ25-C1, IgG1 (Caltag Laboratories); PE-anti-CD20, B-Ly-1, IgG1 (DAKO, Carpinteria, CA); PerCP anti-CD45, 2D1, IgG1 (BD PharMingen, San Diego, CA); FITC anti- κ /PE anti- λ , rabbit polyclonal (DAKO). Washed blood samples were blocked with 2 mg/ml mouse IgG before probing.

Complement opsonization and analyses of patient whole blood

EDTA anticoagulated patient blood was processed as follows: $\sim 0.5\text{ ml}$ of whole blood was washed three times by addition of 4 ml of BSA-PBS, followed by centrifugation at $1260 \times g$ at room temperature (RT) in a swinging bucket centrifuge. The supernatant was then carefully aspirated to spare the buffy coat. The washed cell pellet was reconstituted to its original volume in BSA-PBS. Autologous patient or ABO blood type-matched serum was added, along with RTX and BSA-PBS, to give a final serum concentration of 25% and a final RTX concentration of $10\text{--}25\text{ }\mu\text{g/ml}$. Alternatively, patient sera containing infused RTX, but low in complement titer, were supplemented with autologous or matched sera with full complement activity. One volume of reconstituted washed cell pellet was mixed with one-half volume of patient serum containing RTX, and then either one-half volume of BSA-PBS (25% serum final) or one-half volume of serum with normal complement titer (50% serum final) was added. After incubation for 30 min at 37°C , samples were washed three times, blocked with mouse IgG, and probed with a mixture of PE anti-CD19, PerCP anti-CD45, Al488 anti-C3b(i), and Al633 anti-RTX. After incubation for 30 min at RT, E were lysed, and the samples were washed, fixed in 1% paraformaldehyde PBS, and analyzed by flow cytometry. The combination of CD19, CD45, and side scattering identified B cells, which were analyzed for bound RTX and C3b(i). Calibrated fluorescent beads (Spherotech, Libertyville, IL) were used to convert fluorescence intensities to molecules of equivalent soluble fluorochrome (MESF) (26).

Immunophenotyping

Washed blood cell pellets, reconstituted in 2 mg/ml mouse IgG in BSA-PBS, were incubated for 30 min at 37°C with or without unlabeled RTX ($50\text{ }\mu\text{g/ml}$). Samples were washed and probed with a mixture of APhCy

anti-CD19, PerCP anti-CD45, and one of the following: Al488 anti-RTX; Al488 RTX (anti-CD20) + PE-anti-CD5; Al488 anti-C3b(i) + PE anti-CD20; FITC anti- κ + PE anti- λ ; Al488 IgG1 isotype control; Al488 IgG2a isotype control; or no addition.

Fluorescence microscopy

Washed blood cell pellets reconstituted in 2 mg/ml mouse IgG in BSA-PBS were incubated for 30 min at RT with a mixture of Al488 anti-C3b(i) and Al594 anti-RTX, processed, and examined with a BX40 fluorescent microscope (Olympus, Melville, NY), equipped with a Magnafire digital camera.

Killing assays

Raji cells or isolated patient PBMC were adjusted to 10^6 cells/ml in medium (RPMI 1640, antibiotics, and FBS) (26), and $100\text{ }\mu\text{l}$ mixed with $25\text{--}50\text{ }\mu\text{l}$ of patient sera and additional reagents, including RTX, complement component C2 (Advanced Research Technologies, La Jolla, CA), NHS, and mAb 3E7. Mixtures were incubated for varying periods at 37°C in 5% CO_2 , and after two washes stained with FITC annexin V and propidium iodide and analyzed by flow cytometry (26). Alternatively, sera deficient in individual complement components (Quidel, San Diego, CA) were examined (10% final concentration) \pm the missing complement protein (Advanced Research Technologies) \pm RTX ($10\text{ }\mu\text{g/ml}$) to determine the role of individual components of the complement pathway in RTX-mediated CDC of Raji cells.

Total complement hemolytic activity (CH50) assays

Sheep E were opsonized with rabbit anti-sheep hemolysin (Sigma-Aldrich, St. Louis, MO). NHS or patient sera were serially diluted, and equal volumes of diluted sera were incubated with opsonized sheep E for 1 h at 37°C . Serum dilutions were examined in duplicate or triplicate, and the complete hemolysis titration curve was used to calculate complement titer (33). An NHS pool was used as a standard (absolute CH50 titer of 280; clinical laboratories at the UVA Hospital) and was normalized to a titer of 100 for each day's experiment. Data were evaluated for significance by Student's *t* test (Sigmastat; Jandel, San Rafael, CA). C2 titers of selected patient sera were determined (compared with a purified C2 standard) by measuring their ability to promote hemolysis of sensitized sheep E in the presence of C2-deficient serum (33).

RTX assays

The RTX concentration in patient sera and plasmas was determined by ELISA or flow cytometry; detailed procedures will be reported separately. In brief, the ELISA was based on capture and development of RTX with rabbit anti-mouse IgG and peroxidase-labeled goat anti-mouse IgG, respectively. Alternatively, Raji cells were incubated with RTX-containing samples or prepared standards, and then washed and developed with Al488 anti-RTX to determine RTX levels.

Western blotting

Isolated patient mononuclear cells were lysed in 1% Triton X-100 buffer on ice (34) and centrifuged at $14,000 \times g$, and the detergent-soluble fraction was subjected to SDS-PAGE on 4–15% gradient gels and transferred onto nitrocellulose. The membranes were blocked with 5% milk, incubated with a 200-fold dilution of rabbit polyclonal anti-CD20 (C terminus specific; LabVision, Fremont, CA) or mouse mAb anti-CD20 (N terminus specific; Santa Cruz Biotechnology, Santa Cruz, CA), washed, and developed using a 10,000-fold dilution of donkey anti-rabbit IgG HRP or sheep anti-mouse IgG HRP, respectively, with ECL-plus as substrate (Amersham Pharmacia Biotech). Finally, the pellets that were insoluble in 1% Triton X-100 were solubilized in 3% Triton X-100 and also examined by Western blotting.

Results

Binding of RTX to CD20⁺ cells consumes complement at high cell burdens in vitro and in vivo

Cells from three CD20⁺ lines were incubated with varying concentrations of RTX in 50% NHS at a concentration of $1 \times 10^5\text{ cells}/\mu\text{l}$ for 1 h at 37°C . Cells were pelleted, and the complement titer of the supernatants was measured (Fig. 1). Previous investigations have demonstrated that binding of RTX to CD20⁺ cells promotes complement activation, as demonstrated by C3b(i) capture (10, 26, 27). The present results indicate that CD20-RTX im-

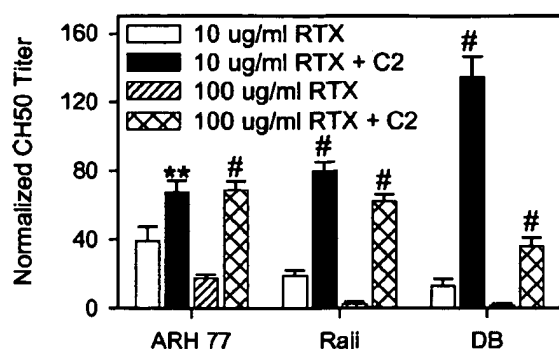


FIGURE 1. Binding of RTX to CD20⁺ cells depletes complement at high cell densities, and addition of C2 restores complement activity. Human B cell lines were incubated in 50% NHS with RTX at 1×10^5 cells/ μ l for 1 h at 37°C. The supernatants \pm C2 were assayed for CH50. Mock incubations without RTX, or without cells, gave no decrease in CH50 (CH50 = 100). Averages and SD of three determinations. The reduction in CH50 upon addition of RTX to sera plus cells was highly significant, $p < 0.001$ in all cases. **, $p < 0.01$; #, $p < 0.001$ for C2 addition vs the comparable control. Representative of two similar experiments.

mune complexes formed on the cells also consume complement at cell densities that are often seen in CLL (7–9) (also, see Table I). At cell densities of 2×10^4 cells/ μ l, we observed moderate complement consumption ($\sim 50\%$ reduction in CH50; data not shown). Complement component C2 is the component at lowest concentration in serum, and is likely to be the first component dissipated during complement activation (35). In fact, addition of C2 alone to these C-depleted sera led to substantial restoration of complement activity (Fig. 1).

Based on these in vitro findings, we measured the complement titer in sera of CLL patients before and after RTX treatment. In four CLL patients with circulating CD20⁺ lymphoma cells, treatment with RTX led to profound loss of complement activity. Individual patients manifested distinct phenotypes with respect to the dynamics of complement depletion and recovery. For example, there was loss of complement activity in serum of patient 1 after the RTX infusion; thereafter, his complement levels did not fully recover until after the 4-wk cycle was completed (Fig. 2A). This pattern of complement depletion was repeated for patient 1 during two additional 4-wk cycles of RTX treatment. Serum of patient 9 lost complement activity after RTX infusion, but complement was almost completely restored 1 wk later (Fig. 2B), and similar patterns were observed for patient 33 (data not shown). Serum of patient 22 also lost complement activity after RTX infusion, but 4 days later, when RTX was no longer demonstrable in the circulation (data not shown), complement levels were restored (Fig. 2C).

After RTX treatment, concentrations of hemolytically active C2 for CLL patients 1, 9, and 33 averaged less than $2 \mu\text{g/ml}$. C2 concentrations for these CLL patients before RTX treatment averaged $25 \mu\text{g/ml}$. In analogy to our results obtained with C-depleted serum supernatants from cell lines, addition of complement component C2 to C-depleted sera substantially restores complement activity (Fig. 2, B and C). In addition, there was virtually no loss ($\leq 33\%$ decrease) of complement activity after RTX treatment in a total of 76 non-CLL patient sera, representing 19 patients. These results suggest that in 4 CLL patients, RTX binds rapidly to accessible CD20⁺ target cells in the bloodstream (and perhaps in the spleen), resulting in rapid complement activation and consumption.

Reduced RTX-mediated cytotoxicity in complement component-depleted or in low complement titer patient sera is restored by addition of complement components or NHS

In view of the reduction in CH50 and depletion of C2 after RTX infusion, we examined the importance of individual complement components in killing Raji cells (Fig. 3A). RTX-mediated killing is modest in sera lacking single complement components; addition of the missing complement component to sera containing RTX markedly increases killing. No additional killing was observed when these components were added to sera in the absence of RTX (data not shown).

We next examined the ability of sera of CLL patients treated with RTX to kill Raji cells. Before treatment, killing by naive patient sera was modest and comparable to that of NHS (10–15%; data not shown). After RTX infusion, sera of CLL patients 1, 8, and 9 had lower cytotoxic activity compared with post-RTX sera of control patients 5, 6, and 10 (Fig. 3B) (NHL, low grade B cell lymphoma, low CD20 CLL, respectively). In these control post-RTX sera, there was no loss of complement activity, and a very high level of Raji cell killing was evident. These sera killed $>90\%$ of Raji cells in a 24-h incubation period, and killing was demonstrable immediately after RTX infusion (serum RTX $>200 \mu\text{g/ml}$), and 1 wk later (serum RTX $>150 \mu\text{g/ml}$), before the next infusion.

Although the levels of RTX were lower in CLL patient sera 1, 8, and 9 1 wk after infusion (13 ± 8 , 91 ± 36 , $3 \pm 1 \mu\text{g/ml}$, respectively), the reduced Raji cell killing potential of the sera, especially immediately after RTX infusion ($>133 \mu\text{g/ml}$ average levels), might be due to reduced complement levels. To test this hypothesis, we supplemented post-RTX CLL patient sera with NHS, or with complement component C2. The results demonstrate that addition of NHS or C2 substantially enhances the efficacy of RTX-mediated killing by CLL sera (Fig. 3C). However, in CLL patient 8, addition of C2 was not as effective; sera of this patient had low complement titers before treatment, and although we have not identified the origin of the defect, our results suggest that it was

Table I. Temporary sequestration of lymphocytes during RTX infusion^a

| Pat. No. | Pretreatment | | During 30 mg of RTX | | Immediately Posttreatment | |
|-----------------|--------------|---------------|---------------------|---------------|---------------------------|---------------|
| | WBC/ μ l | % Lymphocytes | WBC/ μ l | % Lymphocytes | WBC/ μ l | % Lymphocytes |
| 1 ^b | 17,300 | 75 | 4,000 | 37 | 7,400 | 59 |
| 1 ^b | 8,000 | 57 | 2,700 | 20 | 4,500 | 26 |
| 22 | 138,000 | 75 | 51,000 | 90 | 170,000 | 93 |
| 33 ^c | 25,300 | 82 | 6,800 | 64 | 18,000 | 67 |
| 33 ^c | 23,100 | 75 | 8,400 | 49 | 12,600 | 57 |

^a Other data on these samples are presented in Fig. 7; WBC, white blood cells.

^b Patient 1, two different treatment cycles, 3 mo apart.

^c Patient 33, two consecutive treatments, 1 wk apart.

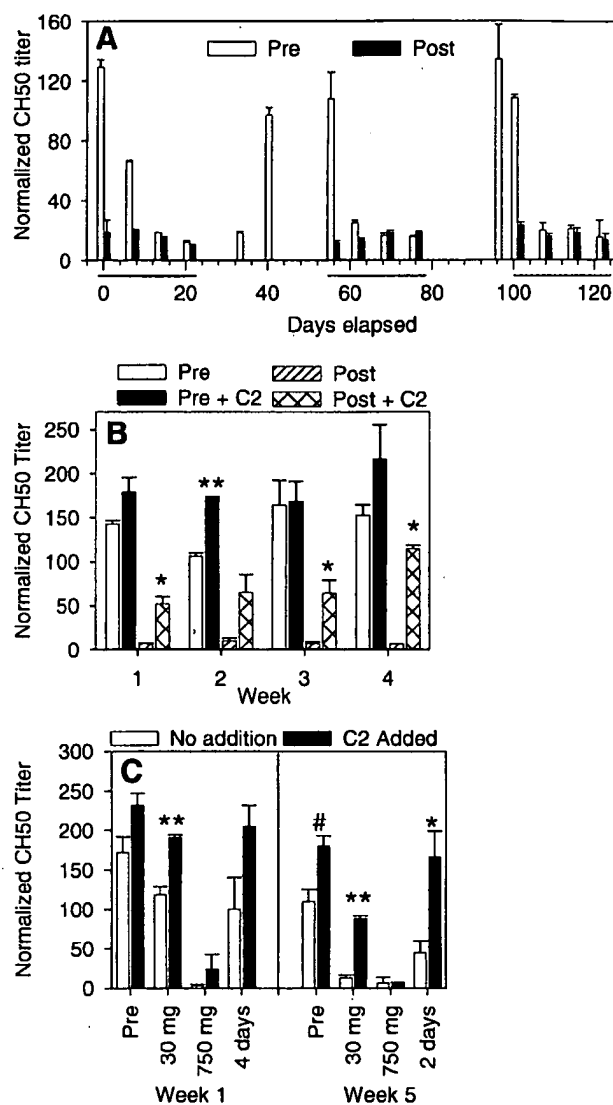


FIGURE 2. Complement is consumed in the bloodstream of CLL patients during RTX treatment. *A*, CH50 of sera from patient 1 before (Pre) and after (Post) RTX treatments. The horizontal lines denote three treatment cycles. CH50 values reported on days 34, 41, and 97 were not associated with RTX treatments. Averages and SD of three replicates. Representative of three independent determinations. *B*, CH50 of sera from patient 9 before (Pre, □) and after (Post, ▤) RTX treatment ± C2 (■ and ▨, respectively). Averages and SD of two replicates, performed in duplicate. *C*, CH50 of sera from patient 22 (□) before, during (30 mg RTX administered), after (750 mg administered), and 4 days (week 1) or 2 days (week 5) posttreatment with RTX for the first and second treatment, 5 wk later. The CH50 was also determined after supplementation with C2 (■). Averages and SD of two replicates, performed in duplicate. *, $p < 0.05$; **, $p < 0.01$; #, $p < 0.001$, for C2 addition vs the comparable control. *A–C*, Compared with naive serum, the reduction in CH50 immediately after RTX infusion was highly significant ($p < 0.001$).

not due to lack of C2. Finally, dose-response experiments indicated that supplementation with 10% NHS was sufficient to recover the maximum killing potential of C-depleted patient sera containing RTX (data not shown).

The *in vitro* binding of RTX to CLL cells in NHS promotes C3b(i) deposition on these cells (26), and we have observed RTX-induced complement activation in the bloodstream of CLL patients (Fig. 2). However, RTX is less effective therapeutically in CLL than in NHL (8, 10, 23), probably due to at least two factors: the

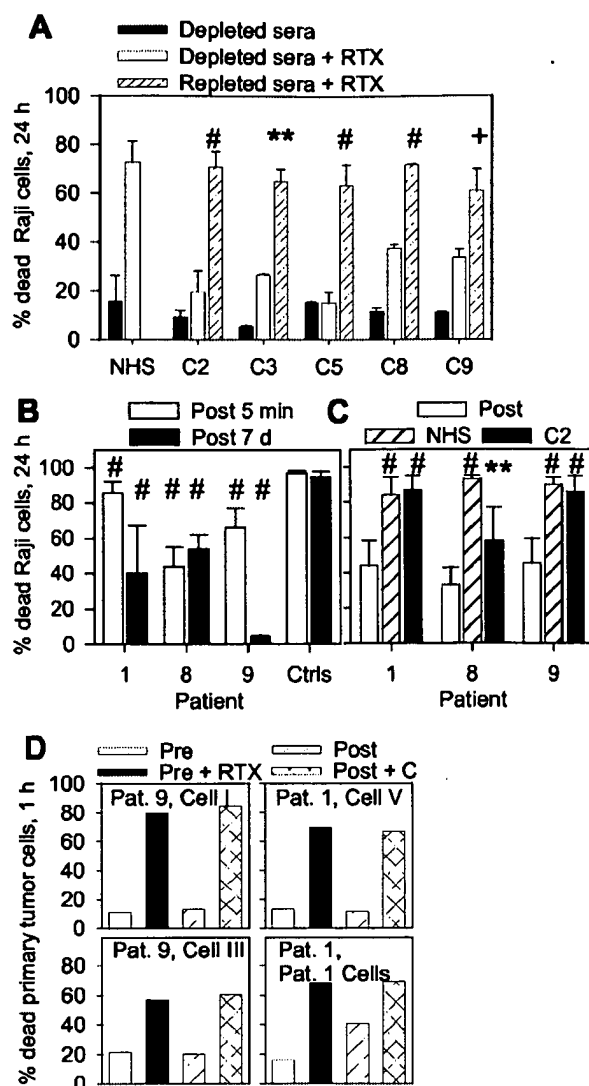


FIGURE 3. Complement promotes RTX-mediated killing of Raji cells and primary CLL cells. *A*, Raji cells were incubated in 10% sera (complement-replete NHS, or human sera depleted of the indicated complement components, ■), or 10% sera + 10 μ g/ml RTX (□), or 10% depleted sera replenished with the deficient component and 10 μ g/ml RTX (▤). Cell viability was determined by flow cytometry. Averages and SD of two or three independent determinations. **, $p < 0.01$; #, $p < 0.001$; +, $p = 0.056$, for depleted serum + RTX vs repleted serum + RTX. *B*, Raji cells were incubated in 33% sera from CLL patients treated with RTX immediately after infusion (□) and 7 days later (■). In *B* and *C* (below), for a given patient, between three and nine different serum samples, obtained during one or more cycles of treatment, were analyzed and averaged. Sera from control patients 5, 6, and 10 retained the ability to kill Raji cells and maintained CH50 titers 5 min and 1 wk after RTX treatment. #, $p < 0.001$ for patients 1, 8, and 9 vs controls. *C*, Raji cells were incubated in sera (16%) from patients 1 ($n = 13$), 8 ($n = 8$), and 9 ($n = 6$), taken immediately after RTX treatment (□) or supplemented with either 16% NHS (▤) or purified C2 (■). **, $p < 0.01$; #, $p < 0.001$ for additional NHS or C2 vs control. *D*, Isolated primary tumor cells from three untreated CLL patients and one previously treated with RTX were incubated for 1 h at 37°C with 16% pre- or post-TX treatment patient sera, ± RTX ± complement, respectively, and assayed for viability by flow cytometry. Pat., Patient.

cells express lower levels of CD20 and they may also up-regulate complement control proteins (8, 10, 23, 36, 37). We have found that a minority (<20%) of CLL patients seen at UVA have B cells that are killed by RTX in NHS *in vitro*. These cells, which tend to

express higher levels of CD20, are also killed by C-replete naive patient sera upon addition of RTX (Fig. 3D, Pre + RTX). Moreover, supplementation of RTX-rich, but C-depleted, sera of RTX-treated CLL patients with a complement source increases their killing of these primary CLL cells (Fig. 3D, Post + C).

mAb 3E7, most specific for C3b(i) covalently deposited on cell surfaces, binds avidly to RTX-opsonized cells in NHS, and can enhance RTX activity in killing cells and in suppressing growth (26). We examined the potential of this mAb to enhance RTX-mediated killing of Raji cells by sera of patients with adequate complement levels. As noted above, in the 24-h killing assay, >90% of Raji cells are killed. Therefore, to allow for clear demonstration of the effects of mAb 3E7, we report the percentage of live Raji cells at 24 h for RTX-containing serum samples examined \pm mAb 3E7 (Fig. 4). In four independent experiments with several different sera, mAb 3E7 clearly enhances the action of RTX in both promoting killing and suppressing growth.

Reduced RTX-mediated C3b(i) opsonization is evident in low complement titer patient sera

The mechanism of action of RTX in vivo may include ADCC, which can be mediated by Fc γ receptors acting in concert with receptors for cell-bound C3b(i) activation products (38, 39). Therefore, in view of the reduction in complement titer observed in CLL patients' sera after RTX treatment, we investigated whether in vitro C3b(i) opsonization of the patient's own cells was compromised. We determined the efficacy of a patient's serum containing RTX to opsonize their cells, based on development with mAbs specific for C3b(i) or for RTX. Addition of RTX to complement-replete sera (either NHS, or pretreatment patient serum) leads to deposition of C3b(i) activation fragments (Fig. 5, Pre, RTX). When sera taken after RTX infusion and subsequent complement consumption are used, although RTX binds to the cells, C3b(i) opsonization is low (Fig. 5, Post). Supplementation of these sera with NHS or with the patient's own pretreatment serum (high in complement activity) fully restores deposition of C3b(i) fragments mediated by RTX (Fig. 5, Post, Pre).

Infused RTX can opsonize cells in the bloodstream

In view of the activation of complement observed after RTX infusion in CLL patients, we examined residual circulating B cells

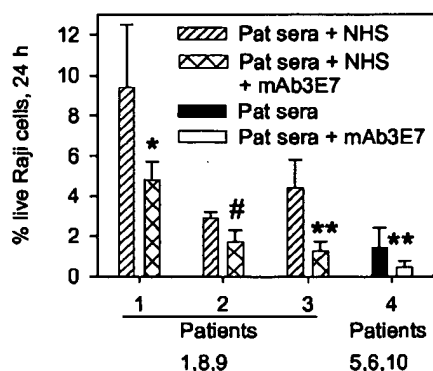


FIGURE 4. mAb 3E7 enhances RTX-mediated killing of Raji cells. Raji cells were incubated in 16% sera from CLL patients 1, 8, and 9, taken immediately after RTX treatment and supplemented with either 16% NHS (▨) or 16% NHS + 10 μ g/ml mAb 3E7 (▩). Sera (16%) from patients 5, 6, and 10 were either not supplemented (■) or supplemented with 10 μ g/ml mAb 3E7 only (□). In each experiment, 4–10 determinations were conducted with sera selected from patients 1, 8, and 9 (experiments 1–3) or patients 5, 6, and 10 (experiment 4). *, $p < 0.05$; **, $p < 0.01$; #, $p < 0.001$ for treatment with mAb 3E7 vs the comparable control. Pat., Patient.

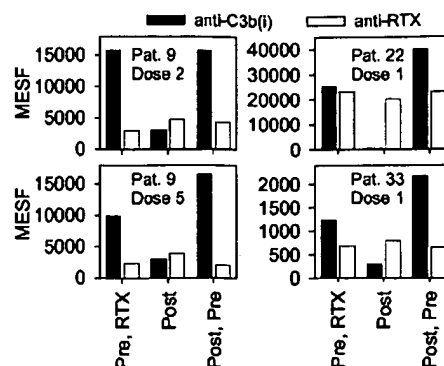


FIGURE 5. Supplementation of patient sera with complement restores RTX-mediated C3b(i) opsonization of autologous B cells. Primary tumor cells from three CLL patients, obtained immediately before RTX treatment, were opsonized for 30 min at 37°C with: autologous pretreatment serum (no RTX, normal complement) + RTX (50 μ g/ml); posttreatment serum (typically >100 μ g/ml RTX, but low in complement); or posttreatment serum (RTX, low complement) + pretreatment serum (no RTX, normal complement). Opsonized cells were washed and probed to reveal bound RTX and deposited C3b(i), and analyzed by flow cytometry. The values for anti-C3b(i) are divided by 10. Background binding for anti-RTX (no RTX added) averaged less than 100 MEF. Pat., Patient.

for bound RTX and/or bound C3b(i) fragments. Based on our previous in vitro studies, we expected that a fraction of these cells would contain RTX colocalized with deposited C3b(i). As infusion of RTX promotes rapid clearance of both normal and malignant B cells 24 h postinfusion (1–6, 19, 40, 41), we analyzed blood samples collected before, 5 min after completion of RTX infusion, and in several cases during RTX infusion, after 30 mg was infused.

Fluorescence microscopy analyses indicate that RTX activated complement and promoted deposition of C3b(i) on circulating cells (Fig. 6). Blood samples of patient 1, taken during and immediately after RTX infusion, had numerous examples of cells and cell aggregates with debris, portions of which showed coincident staining for RTX and C3b(i). Patient 1 was treated again with RTX 1 year later, and we again observed cellular colocalization of RTX and C3b(i). Similar patterns, indicative of uptake of RTX and C3b(i), were evident when the patient's B cells were analyzed by flow cytometry (data not shown). In the case of patient 22, intact cells had discrete sites showing colocalization of C3b(i) and RTX. No such staining was found in comparable samples from either patient before RTX infusion (data not shown).

Treatment with RTX acutely reduces CD20 on circulating cells

The CLL patients had varying, but high levels of lymphocyte counts (Table I), ranging from ~20,000 to >100,000 white blood cells/ μ l, of which at least 75% were lymphocytes. The other patients with B cell lymphomas had few circulating malignant cells and averaged <6,000 white blood cells/ μ l, and of these cells only ~20% were lymphocytes. Although in CLL patients 1, 9, 22, and 33 we generally observed reduction in lymphocyte levels after completion of RTX infusion, final cell counts were sufficiently high that we could examine CD20 levels of residual circulating B cells. In the case of patient 22, although the B cell count decreased after infusion of 30 mg of RTX, there was marked recrudescence of cell counts by the end of the 7-h infusion. Similar patterns were observed for other patient samples, although the levels of recrudescence were lower (Table I). Washed patient bloods were assayed for available CD20 based on probing with either A1488 RTX or another PE anti-CD20 mAb. The results, expressed as the

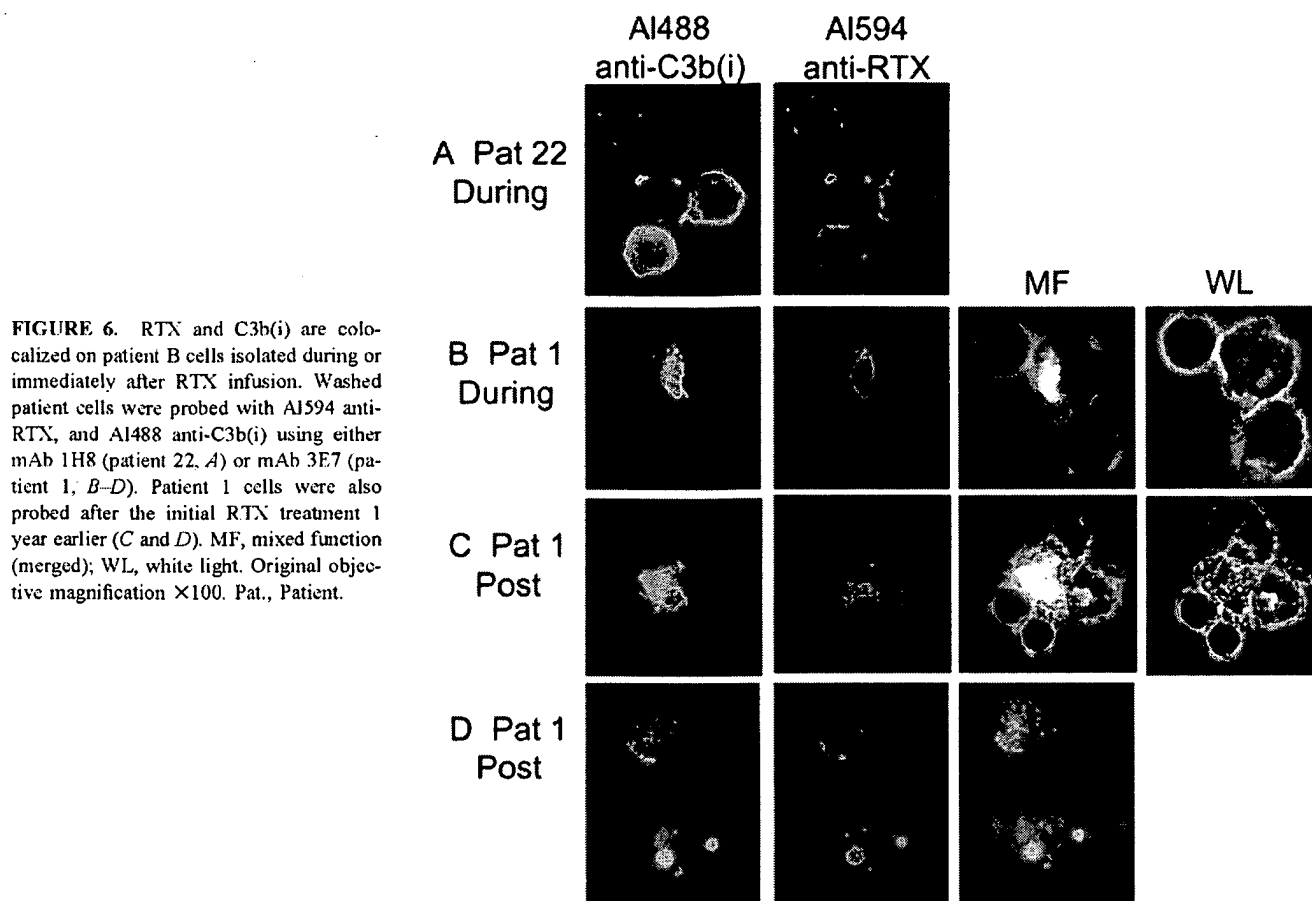


FIGURE 6. RTX and C3b(i) are colocalized on patient B cells isolated during or immediately after RTX infusion. Washed patient cells were probed with AI594 anti-RTX, and AI488 anti-C3b(i) using either mAb 1H8 (patient 22, *A*) or mAb 3E7 (patient 1, *B–D*). Patient 1 cells were also probed after the initial RTX treatment 1 year earlier (*C* and *D*). MF, mixed function (merged); WL, white light. Original objective magnification $\times 100$. Pat., Patient.

MESF ratio for the post-RTX sample vs the pre-RTX sample, indicate that after completion of the RTX infusions, B cells remaining in the circulation have considerably reduced levels of CD20 (Fig. 7*A*, RTX and anti-CD20). Although these results could be interpreted to indicate that CD20 is simply blocked by bound RTX, additional experiments on these blood samples reveal that this is not the case: we reacted washed blood cell pellets, taken before and immediately after RTX treatment, with BSA-PBS (background), or with the patient's post-RTX sera, which typically had final RTX concentrations of $>100 \mu\text{g/ml}$. After incubation, cells were washed and probed with anti-RTX, and the results are again reported as the ratio of MESF bound to post-RTX cells divided by MESF for pre-RTX cells (Fig. 7*A*, ■). The results indicate that RTX binds well to B cells in whole blood taken before RTX infusion; however, RTX binding to post-RTX-treated samples is reduced considerably. This reduced binding cannot be explained by occupancy of CD20 sites by previously bound RTX, as the background signal for bound RTX was much lower in all cases; the levels of RTX bound to washed patient cells after RTX infusion were only slightly higher than the background levels for washed cells taken before RTX treatment. Moreover, after subtraction of this background signal, the anti-RTX signal for washed patient cells after RTX infusion averaged only 5% of the signal for pre-treatment cells reacted with autologous RTX-rich serum. Thus, little residual RTX was bound to the circulating B cells after RTX infusion. Finally, the profound and acute loss of CD20 immediately after the completion of RTX infusion was confirmed by analyses of representative samples by the clinical laboratories at the UVA Hospital (Fig. 7*B*).

The reduction in CD20 levels may be explained by a process in which RTX-CD20 complexes are removed from opsonized B cells

by fixed tissue macrophages (see *Discussion* below). To determine whether CD20 is simply released or internalized due to prolonged reaction with RTX, we incubated primary CLL CD20⁺ cells in washed whole blood reconstituted in 50% autologous serum $\pm 25 \mu\text{g/ml}$ RTX. After 7 h at 37°C (thus modeling the patient infusion paradigm), samples incubated with RTX had modest loss of CD20; compared with controls incubated in the absence of RTX, $86 \pm 28\%$ of the signal was preserved after 7 h ($n = 6$).

Finally, Western blots performed on 1% Triton X-100 extracts of mononuclear cells taken before and after RTX treatment (patient 33) argue that internalization or steric hindrance cannot explain loss of CD20, as posttreatment samples showed a large loss of CD20 (Fig. 7*C*). When the 1% Triton X-100 pellets were solubilized with 3% Triton X-100, very little CD20 (much less than seen in Fig. 7*C*) was found, even after prolonged development (data not shown).

Discussion

Complement and RTX

Binding of RTX to B cells activates complement, and increasing evidence, based on both clinical investigations and a mouse model, indicates that complement may play an important role in the *in vivo* action of RTX (16, 19–28). The present findings reinforce and extend these observations to CLL. Our experiments reveal that at cell levels of $1 \times 10^5/\mu\text{l}$ in 50% NHS, formation of RTX-CD20 immune complexes on several CD20⁺ cell lines depletes complement, and this reaction consumes complement component C2, consistent with activation of the classical complement pathway by these complexes (Fig. 1). We extended this paradigm to RTX treatment of CLL patients with comparable levels of circulating

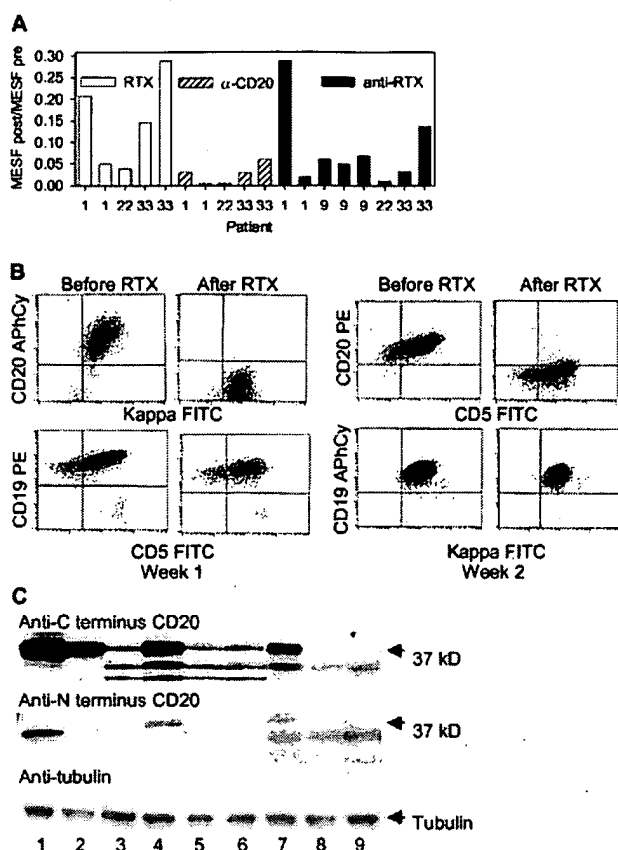


FIGURE 7. Post-RTX treatment patient B cells have substantially reduced CD20. *A*, Washed whole bloods were probed with either Al488 RTX (□) or PE anti-CD20 (▨) and PerCP CD45 and APhCy CD19. The levels of CD20 on CD45⁺, CD19⁺ cells are presented as the MESF signal for posttreatment cells divided by the MESF signal for pretreatment cells. Alternatively, the washed cell pellet was reconstituted in post-RTX treatment autologous patient sera as a source of RTX, incubated at 37°C for 30 min, processed as before, and probed with Al633 anti-RTX, PerCP CD45, and PE CD19 (■). Absolute values for binding to pretreatment cells by Al488 RTX ranged from 35,000 to 540,000 MESF; for PE anti-CD20, from 12,000 to 54,000 MESF. Background binding of Al633 anti-RTX to pre- or posttreatment cells averaged less than 300 MESF. After opsonization with RTX, MESF values for Al633 anti-RTX ranged from 500 to 15,000. Multiple determinations for patients 1, 9, and 33 were at different times during treatment. Blood samples for patient 9 were only obtained pre- and postinfusion. White blood cell counts (per μ l) for 3 consecutive wk of treatment of patient 9 were 25,000, 22,000, and 14,000; the corresponding post-samples were 5,000, 6,000, and 3,200, respectively. *B*, Blood samples from patient 33, before and after RTX infusion, were analyzed by the clinical laboratories at the UVA hospital. The PerCP CD45⁺ cells were κ ⁺, CD5⁺, and CD19⁺ before and after RTX treatment, but CD20 was completely lost immediately after RTX treatment. Representative of three other determinations on other patients. *C*, Immunoblots of 1% Triton X-100 cell extracts (50 μ g protein/lane) from 3 consecutive wk of treatment (patient 33). Pre-RTX, lanes 1, 4, and 7; after 30 mg of RTX, lanes 2, 5, and 8; immediately post-RTX, lanes 3, 6, and 9; each set corresponds to weeks 1, 2, and 3, respectively. The bands below 35 kDa in the top and middle panels may correspond to partial digestion products of CD20. The C terminus and N terminus Western blots were performed a total of four and two times, respectively, with very similar results.

CD20⁺ B cells ($\sim 2 \times 10^4$ to $>1 \times 10^5/\mu$ l), and we find that RTX infusion promotes complement consumption and depletion of C2 (Fig. 2).

Although in one case (patient 22, week 2) complement was consumed after infusion of only 30 mg of RTX, in most cases we did

not observe complement consumption until after the infusion was complete, when a total of ~ 700 mg had been infused. It is likely that complement consumption in the other patients would have been demonstrable before completion of the infusion, but study design limited the number of blood samples available for analysis. The amount of RTX infused should have been more than sufficient to saturate CD20 sites on the circulating cells and promote complement activation. For example, after infusion of 70 mg of RTX ($\sim 1/10$ of a full infusion), the initial concentration of RTX within an intravascular volume of 4 L would be 17 μ g/ml. If the cell count were $1 \times 10^5/\mu$ l, this would correspond to a ratio of $>600,000$ RTX molecules per cell, which would be more than enough to bind to all CD20 molecules on the CLL cells, which vary from $<5,000$ to $\sim 100,000$ per cell (10, 23, 27, 37). Moreover, dose-response binding experiments have demonstrated that the affinity of RTX for CD20 is sufficiently high that 10 μ g/ml RTX will saturate binding (24, 27, 42). Finally, we note that in the *in vitro* model system (Fig. 1) we observed consumption of complement at RTX concentrations of 10 and 100 μ g/ml, for cell inputs of 1×10^5 cells/ μ l.

The entire classical complement cascade appears to be required for full cytotoxic activity of RTX against Raji cells (Fig. 3A), and addition of C2 to complement-depleted patient sera restores complement activity as defined by the CH50 assay. Furthermore, addition of C2, pre-RTX patient serum, or NHS to complement-depleted CLL patient sera containing RTX restores B cell-killing activity (Fig. 3, B and C). Although most of the reconstitution-killing assays were performed with Raji cells, we were able to show that addition of a complement source to complement-depleted patient sera containing RTX enhanced RTX-mediated killing of primary CLL cells (Fig. 3D). Therefore, we suggest that if complement is required to promote killing of RTX-opsonized cells, then use of C2, or compatible fresh frozen plasma as a complement source, may enhance the action of RTX in patients with reduced or depleted complement levels.

We used a similar approach to demonstrate that RTX-mediated *in vitro* deposition of C3b(i) fragments on patients' CD20⁺ cells could be restored by supplementation of their RTX-containing serum with either NHS or the patients' complement-replete sera, taken just before RTX infusion (Fig. 5). Restoration of deposition of C3b(i) on target cells may increase the immunotherapeutic action of RTX, even when complement-mediated lysis does not occur. Several lines of evidence suggest that recognition of RTX-opsonized cells by Fc γ receptors on phagocytic cells promotes ADCC and contributes to RTX immunotherapeutic action (10, 15–18). Opsonization of IgG-containing target cells with C3b activation products enhances Fc γ receptor-mediated phagocytosis of cells by both neutrophils and monocytes (38, 39). This enhancement is based on synergistic interaction between complement and Fc γ receptors on the phagocytic cell. Czuczman et al. (43) reported, in a mouse lymphoma model, that up-regulation of CD11b (a subunit of CR3, specific for iC3b) enhanced RTX-mediated ADCC, and we suggest that deposition of C3b activation products on RTX-opsonized cells will enhance ADCC. Thus, with respect to the continuing controversy regarding the *in vivo* mechanism of action of RTX, it appears that complement-promoted lysis, and cellular cytotoxicity, mediated by both complement receptors as well as Fc γ receptors, play important roles.

Dynamics of B cell opsonization and clearance

Previously, we reported, based on *in vitro* studies and a monkey model, that complement activation induced by binding of RTX to

CD20⁺ cells promotes deposition of large numbers of C3b activation products colocalized with cell-bound RTX (26). We endeavored to replicate this finding in RTX-treated patients, by isolating and identifying RTX-opsonized cells containing bound C3b(i). In most cases, we found few RTX-opsonized cells in the circulation after RTX treatment. However, we were able to identify cells opsonized with RTX and C3b(i) in the circulation of two RTX-treated CLL patients (Fig. 6). The fact that RTX and C3b activation products were colocalized on the cells or cellular debris is consistent with our previous observations and provides additional evidence that cell-bound RTX is an important site for capture of nascent C3b in vivo (26).

A proposed mechanism for acute RTX-mediated loss of CD20 in CLL

RTX treatment led to reduction in lymphocyte counts, and the reduction in lymphocytes was demonstrable after infusion of only 30 mg of RTX (Table I). However, after RTX infusion was completed (4–7 h later), often there was an increase in lymphocyte counts (compared with the number observed after 30 mg of RTX), and at this time B cells had considerably reduced levels of CD20 (Fig. 7). These observations, taken in the context of work reported by Schreiber and Frank (44) and Griffin et al. (30), suggest a mechanism by which the RTX-opsonized cells were processed.

Studies of the clearance of IgM/C3b-opsonized ⁵¹Cr-labeled E revealed that these cells are rapidly removed from the bloodstream by liver macrophages that have receptors specific for C3b or iC3b (44). However, a sizeable fraction of opsonized E is later released back into the circulation after C3b fragments bound to the cells are degraded to C3dg. Thus, there is precedence for temporary sequestration of opsonized cells, followed by proteolytic processing steps that allow cells to be released. In studying phagocytosis of IgG-opsonized lymphocytes, Griffin et al. (30) reported that capping of IgG on targeted lymphocytes could lead to removal of the cap and thus “prevent the destruction of these cells by macrophages.” Binding of RTX to CD20⁺ cells may induce rearrangement and cross-linking of CD20, and these RTX-CD20 complexes can be clustered by action of macrophage Fcγ receptors (11). Griffin et al. postulated that “phagocytic cells may clear” (Ab-opsonized) “abnormal surface determinants from these (neoplastic) cells, leaving them no longer recognizable as abnormal and thus able to proliferate within the host” (30).

We believe that the CD20-depleted B cells, which we observed in the circulation of CLL patients after RTX treatment, are lymphoma cells that had previously bound RTX and C3b(i). These cells had reduced CD20 and contained very little bound RTX (Fig. 7A). Western blotting experiments confirmed loss of CD20 (Fig. 7C), and our findings and other reports indicate it is unlikely the cells spontaneously internalized and/or shed CD20 after ligation by RTX (1, 11, 45). Thus, our results suggest that these cells were temporarily sequestered by phagocytic cells in the liver and/or spleen, where the complexes of CD20, RTX, and C3b(i) were removed, thus allowing the CD20-depleted lymphocytes to return to the circulation. Foran et al. (46) reported the case of a mantle cell lymphoma patient who died of splenic rupture following RTX treatment. The patient had a high burden of circulating cells, and these cells lost CD20 after RTX treatment. We suggest that the mechanism we have proposed may have led to acute CD20 loss in this patient.

Studies in monkey models and more recent reports in humans suggest that normal B cells are rapidly depleted from the circulation upon treatment with RTX (19, 26, 40, 41, 47). We are unaware of any reports suggesting loss of CD20 from normal cells and/or recrudescence of the cells after RTX treatment, and the reason may

simply be that the cell burden is considerably lower than that found in CLL.

Therapeutic implications

It is generally believed that one of the reasons for the efficacy of RTX as an immunotherapeutic agent is its stable binding to CD20 on B cells with little internalization or release (1, 11, 45). Although the mechanism of action of RTX is still under investigation, its Fc region is required to promote complement activation and/or ADCC in the immunotherapy of NHL (19). Our experiments suggest that in CLL, processing of RTX-opsonized cells in the circulation allows for more complex mechanisms to operate. In particular, removal of RTX-CD20 complexes by the MPS may allow CLL cells to escape and nullify the RTX immunotherapeutic potential. Use of RTX as a single agent has been less effective in CLL than in the lymphomas (8, 10, 23), and this escape mechanism may be an important underlying factor. Use of other anti-CD20 mAbs, which can kill B cells as F(ab')₂, may provide an approach for targeting circulating CD20⁺ cells in CLL (42). Alternatively, it should be possible to engineer RTX to activate complement, but not bind to Fcγ receptors (48). Although such an engineered molecule may not be appropriate for the treatment of NHL, it may have therapeutic efficacy in CLL.

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RAPID COMMUNICATION

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V. Diehl · A. Engert

Rapid tumor lysis in a patient with B-cell chronic lymphocytic leukemia and lymphocytosis treated with an anti-CD20 monoclonal antibody (IDEC-C2B8, rituximab)

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Summary In this report we present a patient with B-cell chronic lymphocytic leukemia who developed an acute tumor lysis syndrome after administration of the human anti-CD20 antibody IDEC-C2B8 (RITUXIMAB) in standard dose of 375 mg/m². IDEC-C2B8 has been demonstrated to have only mild and tolerable side effects in patients with follicular lymphoma. In these trials patients with lymphocytosis >5000/μl were excluded. Physicians must be aware of this hitherto unreported phenomenon in patients with high CD20-positive blood counts.

Key words Chronic lymphocytic leukemia · IDEC-C2B8 · Rituximab · Tumor lysis syndrome · Immunotherapy

Introduction

Clinical trials with the chimeric human monoclonal anti-CD20 antibody IDEC-C2B8 [1] (rituximab) demonstrated remission rates up to 50% in relapsed low-grade follicular non-Hodgkin's lymphoma [2, 3]. Efficacy and safety in the treatment of chronic lymphocytic leukemia (CLL) and other blood-born tumors has not been investigated yet. Here we report on a 26-year-old woman with B-CLL who experienced a rapid reduction of circulating malignant cells accompanied by severe side effects after her first rituximab infusion.

Case report

In October 1997, a 26-year-old female patient with progressive low-grade B-cell lymphoma was admitted to

our hospital. She had been heavily pretreated including 12 cycles of intensive chemotherapy and high dose chemotherapy with peripheral stem cell support. She had enlarged cervical and abdominal lymph nodes, hepatosplenomegaly and bone marrow infiltration. A leukocytosis of $111.9 \times 10^9/L$ with 97% small malignant lymphocytes was noticeable, phenotypically resembling B-cell chronic lymphocytic leukemia (CD5+, CD10-, CD19+, CD23+, CD25+). The CD20 surface marker was expressed on 100% of these cells. As a result of clinical reevaluation, a treatment with the anti-CD20 antibody rituximab was initiated.

After prophylactic hydration, as well as administration of 1000 mg acetaminophen and 300 mg allopurinol, treatment was begun with a predose of 50 mg antibody. The patient complained about moderate scratching sensations in her throat, chills, and a moderate rise in body temperature 90 min after this test infusion. Following pethidine, she recovered quickly, and the remaining 550 mg of the planned dose (375 mg/m²) was given over four and one-half hours. Shortly after the infusion was completed, chills occurred again and fever up to 39.7°C developed. The pulse increased to 124/min. The patient complained about nausea and vomiting. A blood count revealed a sharp decline in leukocytes from $111.9 \times 10^9/L$ to $24.0 \times 10^9/L$ and a drop in platelets from $137.0 \times 10^9/L$ to $35.0 \times 10^9/L$. The plasma prothrombin time fell from 92% to 65%, and serum LDH activity rose from 464 U/L to 793 U/L.

During the following 24 hours, the clinical condition and laboratory parameters of the patient further deteriorated (see Figure), resembling an acute tumor lysis syndrome with disseminated intravascular coagulation. Serum LDH rose to >2000 U/L; plasma prothrombin time and platelets continued to drop to 47% and $23 \times 10^9/L$, respectively. The plasma D-dimers rose from 4.8 mg/L to 42.2 mg/L; serum transaminase activities were moderately increased (Figure). Importantly, complement factors were undetectable 7 hours after the beginning of the infusion. The patient was intensively treated with forced diuresis including furosemide, bi-

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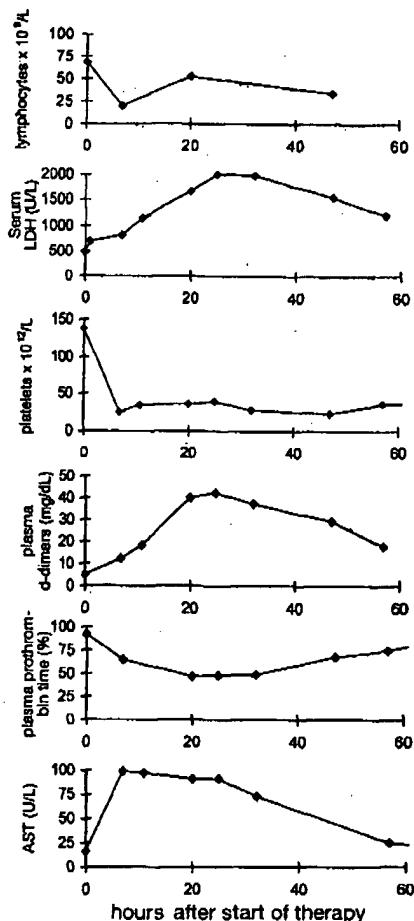


Fig. 1. Patients laboratory parameters during first rituximab infusion, indicating and episode of rapid tumor lysis with drop of lymphocytes, increased serum lactate dehydrogenase (LDH) activity abnormalities in platelet counts, coagulation parameters and serum aspartate aminotransferase (AST) activity

carbonate, calcium, potassium, platelet transfusion as well as ondansetron. As a result, the clinical performance status gradually improved from day two onwards, and laboratory parameters began to normalize. Three further infusions of rituximab were administered in full dose on days 8, 15, and 22 without clinical problems. The patient's leukocyte count subsequently normalized ($8.8 \times 10^9/L$) for 3 weeks whereafter she showed signs of progressive disease, requiring salvage chemotherapy.

Discussion

Fast but transient clearance of circulating lymphoma cells without major side effects using a monoclonal antibody was reported by Nadler et al. [5] in a patient with diffuse, poorly differentiated lymphocytic lymphoma (DPDL) and a WBC of $110.000 \times 10^9/L$. In patients

with chronic lymphocytic leukemia, elimination of malignant cells from the blood stream has been observed using another human monoclonal antibody, Campath-1H (anti-CD52) [6]. The effector mechanisms of Campath-1H include complement-mediated lysis and cellular cytotoxicity and are very similar to those employed by rituximab [1]. Since complement factors dropped to undetectable levels in our patient within seven hours, it might be speculated that the complement-mediated cell lysis played a decisive role in triggering the acute tumor lysis observed.

Although the major non-hematological side effects such as nausea, fever, rigor or hypotension are similar for rituximab and Campath-1H, none of 29 CLL patients treated with Campath-1H showed signs of rapid tumor lysis [7]. One possible explanation is the different schedule used: Campath-1H was administered three times weekly at initial doses of 3 or 10 mg which were escalated to 30 mg. The recommended standard dose of 375 mg/m^2 for rituximab was established in patients with follicular lymphoma and lymphocyte counts of less than $5.0 \times 10^9/L$. Thus, this dose might be too high for the treatment of patients with substantial peripheral tumor load. Alternatively, high peripheral tumor cell counts must be reduced using cytostatic drugs prior to administration of rituximab.

Recently, we have treated six additional B-CLL patients and one patient with a leukemic mantle cell lymphoma with rituximab. The clinical side effects were minor in three patients with lymphocyte counts of $0.2 \times 10^9/L$, $6.6 \times 10^9/L$, and $9.4 \times 10^9/L$, respectively. Signs of acute tumor lysis and NCI grade III and IV toxicities occurred in patients with marked lymphocytosis who had $30.7 \times 10^9/L$, $60.8 \times 10^9/L$, $69.8 \times 10^9/L$, $108.5 \times 10^9/L$, and $294.3 \times 10^9/L$ lymphocytes, respectively.

When treating patients with CLL and marked lymphocytosis with the monoclonal antibody rituximab, physicians need to be aware of the risk of hitherto unreported acute tumor lysis and intravascular coagulation.

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Review

Key Issues in the Treatment of Chronic Lymphocytic Leukaemia (CLL)

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The outcome of the treatment of chronic lymphocytic leukaemia (CLL) has improved little over the past 30 years. The recent introduction of purine analogues, particularly fludarabine, may change this situation. These agents are highly effective and generally well tolerated. They raise the possibility of improved disease-free survival and allow appropriate patients to be considered for bone marrow transplantation (BMT). Randomised clinical trials are needed to establish the roles of purine analogues and other novel agents in improving the survival of CLL patients. These trials should use consistent diagnostic and assessment criteria to allow for the clinical heterogeneity of CLL.

Key words: chronic lymphocytic leukaemia (CLL), purine analogues, prognostic factors

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INTRODUCTION

CHRONIC LYMPHOCYTIC LEUKAEMIA (CLL) is often described as an "indolent" disease, but this is not the case in at least 50% of patients [1]. Examination of survival curves shows that many patients die early, and the absence of a plateau shows that CLL is usually not cured (Figure 1). It is also important to consider

that many patients die of causes not directly related to CLL. The overall median survival is approximately 6 years, and this has changed little over the past 30 years [1]. Recent advances in the diagnosis and treatment of CLL may improve this situation [2]. This article examines key issues that need to be addressed to optimise the treatment of this disease.

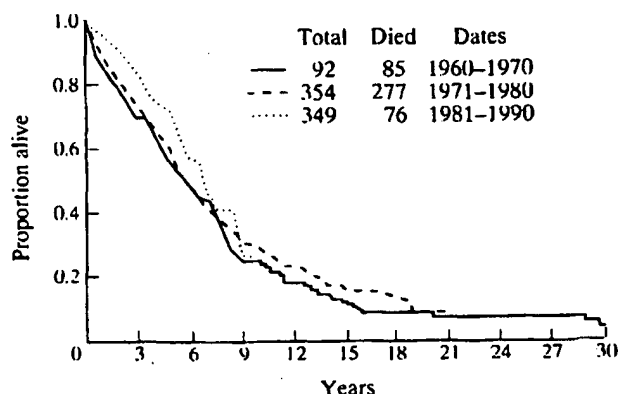


Figure 1. Survival of patients with untreated CLL by decade (MD Anderson Cancer Center). Reproduced with permission from Keating M. Chemotherapy of chronic lymphocytic leukaemia. In Cheson BD, ed. *Chronic Lymphocytic Leukemia. Scientific Advances and Clinical Developments*. New York, Marcel Dekker, 1993, 297-336.

DIAGNOSIS OF CLL

The diagnosis of CLL depends on the detection of lymphocytosis in the blood and bone marrow. Threshold peripheral blood lymphocyte counts of $5 \times 10^9/l$ and $10 \times 10^9/l$ have been recommended by the National Cancer Institute Working Group on CLL (NCIWG) [3] and the International Workshop on CLL (IWCLL) [4], respectively. Differential counts of bone marrow aspirates must reveal that over 30% of all nucleated cells are mature-appearing B-lymphocytes. Examination of peripheral blood films is useful to confirm that the malignant cells are lymphocytes, which usually have distinct nuclear chromatin clumping.

Immunophenotyping is an essential tool for distinguishing CLL from other B-cell or T-cell disorders [5]. Recent results indicate that no single immunological marker is completely specific for CLL, but examination of several markers reveals a profile that objectively distinguishes CLL from other disorders in the majority of cases [6].

PROGNOSTIC FACTORS

The course of CLL varies widely between patients, therefore, prognostic factors must be evaluated to predict the course of the

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disease and select the most appropriate treatments. The principle prognostic factors identified to date are summarised below.

Age

Older patients have a poorer prognosis than younger patients [7–10]. A recent survey has indicated that when non-CLL causes of death are excluded, younger patients (< 50 years) have no better survival than older patients [11]. This result suggests that prognostic indicators are no different in the two age groups, and that the shorter survival in older patients is a consequence of unrelated diseases. The median survival in patients aged < 50 years, who account for 5–7% of patients, is at least 20 years shorter than comparable controls [8, 11]. These observations justify the evaluation of more intense treatments in younger patients with advanced disease and other poor prognostic factors.

Sex

Women with CLL have a better prognosis than men, even after correction for other factors including age and stage [10, 12]. The reason for this is not fully understood.

Clinical stage

Staging, using either the Rai or Binet systems, is the most important prognostic factor for survival in CLL (Figure 2) [10, 13, 14]. The patients with the worst prognosis are those in Binet stage C, which is equivalent to Rai stages III and IV. The IWCLL has proposed a classification that combines the Rai and Binet systems and identifies distinct subgroups within the stages [3]. This system has not been widely adopted, although the best prognosis within stage A is the subgroup A (0).

Lymphocyte count and doubling time

The NCIWG and the IWCLL have defined lymphocytosis at thresholds of $5 \times 10^9/l$ and $10 \times 10^9/l$, respectively [2, 3], although it has been suggested that a diagnosis of CLL could be made with lower counts [15]. Immunophenotyping to confirm the diagnosis is essential, particularly in cases with low lymphocyte counts. A high lymphocyte count correlates with advanced clinical stage and a poor prognosis [6, 12].

In patients with Binet stages A and B, a lymphocyte doubling time of less than 12 months correlates with more rapid progression and shorter survival [16, 17]. The NCIWG regards a

doubling time of less than 6 months as an indication for treatment [2]. Data from the Medical Research Council (MRC) CLL3 trial appear to confirm the strong prognostic value of a lymphocyte doubling time < 12 months compared with > 12 months (D. Catovsky, unpublished observations).

Bone marrow pattern

A trephine biopsy should be a standard investigation in CLL. A diffuse or packed pattern of bone marrow infiltration confers a worse prognosis than a non-diffuse (nodular, interstitial or mixed) pattern. As a diffuse pattern is correlated with a more advanced clinical stage, the independent significance of the bone marrow pattern has not been shown in all studies [18–20]. This information is more important in patients with intermediate prognosis, such as Binet stage B. In this group, a packed bone marrow identifies the group with worse prognosis [18].

Chromosomal abnormalities

Chromosomal abnormalities, most commonly trisomy 12 and 13q deletions, have been detected in approximately 50% of patients with CLL in whom suitable metaphases could be obtained. Cytogenetic analysis is not a routine investigation in CLL and it is not easy to obtain suitable metaphases. In recent years, therefore, fluorescence *in situ* hybridisation (FISH) techniques have been used to detect trisomy 12 in interphase nuclei [21]. A recent investigation, in which FISH and immunophenotyping were performed simultaneously in single interphase cells, showed that trisomy 12 is a secondary event during the leukaemic transformation of CLL and develops in an already established neoplastic B-cell population [22]. Trisomy 12 appears to correlate with worse prognosis, either because it is associated with a typical morphology, particularly CLL-PL [21] and/or because it confers a growth advantage on the malignant cells [22]. A high number of chromosomal abnormalities also predicts a poorer outcome [23–25].

Oncogenes

Activation of oncogenes has, so far, been identified in only a small proportion of patients with CLL. Expression of *bcl-2*, found in 5–10% of patients [26], produced an oncoprotein that inhibits apoptosis in lymphocytes [27] and may confer resistance to cytotoxic agents [28]. The alteration of the *bcl-2* oncogene in CLL is distinct from that seen in follicular lymphoma [29].

Response to treatment

A good response to treatment is associated with longer survival independently of age, sex or disease stage [10]. As a good response is associated with other favourable factors, such as early clinical stage and no previous treatment, the value of this parameter could only be assessed in randomised trials.

DEFINING RESPONSE TO TREATMENT

The NCIWG [3] and the IWCLL [4] have published guidelines to facilitate comparisons between clinical trials in CLL in different centres (Tables 1 and 2). The criteria used in MRC trials 1, 2 and 3 are similar to the NCI criteria.

The NCI criteria for a complete response (CR) allow the persistence of nodules in the bone marrow. This response has been called a "nodular CR" (nCR) to distinguish it from a CR with a normal bone marrow. In one series, patients with a nCR after fludarabine had a shorter time to disease progression than patients with a CR, but their survival was not significantly different [30]. As persistent nodular infiltration predicts earlier

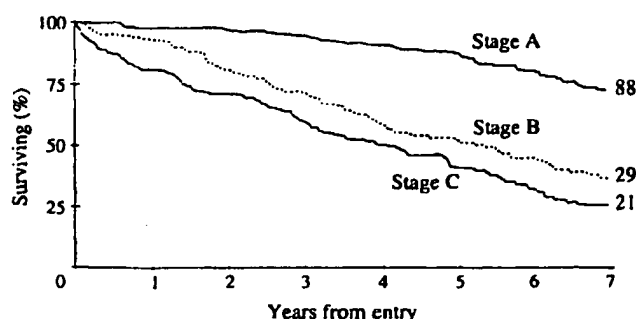


Figure 2. Survival of patients with CLL by Binet/International stage entered in MRC CLL 1 trial [10]. Only CLL-related deaths were considered. Numbers shown indicate the number of patients alive 7 years after entry into the study. Reproduced with permission from Catovsky D, Fooks J, Richards R. Prognostic factors in chronic lymphocytic leukaemia: the importance of age, sex and response to treatment in survival. A report from the MRC CLL 1 trial. *Br J Haematol* 1989, 72, 141–149.

Table 1. NCI guidelines for CLL [11]*

| | |
|--|---|
| Diagnosis | |
| Lymphocytes | $> 5 \times 10^9/l$ |
| Atypical cells (e.g. prolymphocytes) | $< 55\%$ |
| Duration of lymphocytosis | ≥ 2 months |
| Bone marrow lymphocytosis | $\geq 30\%$ |
| Staging | Modified Rai, correlate with Binet |
| Eligibility for trials | <p>"Active disease" (loss of $\geq 10\%$ of body weight in last 6 months; extreme fatigue; fever $> 100.5^\circ F$ for ≥ 2 weeks unrelated to infection; night sweats; anaemia, thrombocytopenia; auto-immune anaemia and/or thrombocytopenia not responding to steroids)</p> <p>Massive splenomegaly or lymphadenopathy</p> <p>Progressive lymphocytosis with an increase of $> 50\%$ in 2 months or doubling time < 6 months</p> |
| Criteria for complete response | |
| Physical examination | Normal |
| Symptoms | None |
| Cell count | Lymphocytes $\leq 4 \times 10^9/l$, neutrophils $\geq 1.5 \times 10^9/l$, platelets $> 100 \times 10^9/l$ |
| Haemoglobin | $> 11 g/dl$ (untransfused) |
| Bone marrow lymphocytes in aspirates | $< 30\%$ |
| Duration | ≥ 2 months |
| Criteria for partial response (used in MRC CLL trials) | |
| Physical examination | $\geq 50\%$ decrease in nodes and/or liver/spleen |
| Plus one of: | Neutrophils $\geq 1.5 \times 10^9/l$, platelets $> 100 \times 10^9/l$, haemoglobin $> 11 g/dl$ or $> 50\%$ improvement |
| Duration | ≥ 2 months |
| Criteria for progressive disease | |
| Physical examination (nodes, liver, spleen) | $\geq 50\%$ increase or new |
| Circulating lymphocytes | $\geq 50\%$ increase |
| Other | Richter's syndrome |
| Criteria for stable disease | All others |

*Reproduced with permission from Molica S, Brugiatelli M, Callea V, *et al.* Comparison of younger versus older B-cell chronic lymphocytic leukemia patients for clinical presentation and prognosis. A retrospective study of 53 cases. *Eur J Haematol* 1994, 52, 216-221.

relapse, nCR might better be designated "nodular partial response" (nPR).

Two-colour flow cytometry, immunophenotypic analysis, FISH and the polymerase chain reaction may help to assess residual disease in patients with a clinical CR [31-33]. In a recent study on 22 patients in clinical CR, residual disease, detected by light chain restriction dual CD5/CD9 staining and Ig gene rearrangement, was associated with a shorter disease-free interval than in patients with negative findings on these tests [34].

CURRENT TREATMENTS FOR CLL

Indications for treatment

Patients with CLL can be stratified into three groups:

- (i) high risk (median survival 1.5-5 years): Binet stage C; Rai III or IV
- (ii) intermediate risk (median survival 5-7 years): Binet stage B; Rai I or II
- (ii) low risk (median survival > 10 years): Binet stage A; Rai 0.

High risk patients suffer frequent complications, so should be

treated as soon as they have been fully evaluated. At diagnosis, most patients fall into the intermediate or low risk groups. These individuals can follow an indolent course, in which CLL progresses slowly and the patient remains asymptomatic for a long period. Patients with indolent Rai 0 or Binet A CLL (smouldering CLL) can survive as long as age- and sex-matched controls. The active disease can be manifested by increasing lymphocytosis and other signs and symptoms (e.g. lymphadenopathy, anaemia, thrombocytopenia, infections), in a variable proportion of patients. Randomised trials have shown that administration of chlorambucil to stage A patients delays progression, but does not improve survival compared with delaying treatment until progression occurs, and might even be harmful [35, 36]. Low or intermediate risk patients may be followed without treatment until signs of disease activity, or adverse prognostic factors, are observed. In practice, stage B patients are treated, although a randomised trial of early or delayed treatment in this group could be considered. Even patients with indolent disease must be assessed regularly because some of them will progress [37]. It is in this group that determination of the lymphocyte doubling time may be important.

Table 2. IWCLL guidelines for CLL [12]*

| | |
|--------------------------------------|--|
| Diagnosis | |
| Lymphocytes | $\geq 10 \times 10^9/l$ + B phenotype or bone marrow involved; $< 10 \times 10^9/l$ + B phenotype and bone marrow involved |
| Atypical cells (e.g. prolymphocytes) | Not stated |
| Duration of lymphocytosis | Not stated |
| Bone marrow lymphocytosis | $> 30\%$ |
| Staging | IWCLL |
| Eligibility for trials | Stage A: lymphocytes $> 50 \times 10^9/l$, and/or doubling time < 12 months and/or diffuse marrow Stage B, C: all patients |
| Criteria for complete response | |
| Physical examination | Normal |
| Symptoms | None |
| Lymphocytes | $< 4 \times 10^9/l$ |
| Neutrophils | $> 1.5 \times 10^9/l$ |
| Platelets | $> 100 \times 10^9/l$ |
| Haemoglobin | Not stated |
| Bone marrow lymphocytes in aspirates | "Normal", allowing nodules or focal infiltrates |
| Duration | Not stated |
| Criteria for partial response | Downshift in stage (cell counts not stated) |
| Duration | Not stated |
| Criteria for progressive disease | Upshift in stage |
| Criteria for stable disease | No change in stage |

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First-line treatment

Chlorambucil is the standard first-line treatment for CLL. It reduces the lymphocyte count in approximately two-thirds of patients and, in a smaller proportion, reduces spleen size and improves platelet counts and haemoglobin [38–41]. Chlorambucil is given orally and it is generally well tolerated. Many centres combine chlorambucil with prednisone, although there is no evidence that addition of this drug, or prednisolone [36], confers any survival benefit. Chlorambucil plus prednisone induces CRs in approximately 25% of patients and PRs in approximately 50% [42–48]. Chlorambucil has been given daily in the past; lately, intermittent dosing has been used more commonly. This modality has similar efficacy and is less toxic [41]. The schedule currently used in the MRC CLL3 trial is chlorambucil, 10 mg/m² daily for 6 days, repeated monthly. High dose continuous chlorambucil has been reported to give greater response rates and longer survival than intermittent chlorambucil plus prednisone in stage A and B patients [49]. This regimen could be tested further, but in a properly randomised trial.

Cyclophosphamide can be used alone in patients who cannot tolerate chlorambucil, but there is no good evidence for its single agent activity. It is used mostly in combination regimens (see below).

Prednisone given alone has only modest effects in the primary treatment of CLL [38, 50], but it is valuable in the treatment of autoimmune anaemia or thrombocytopenia [15]. In the early treatment of stage C (or stage III–IV) disease, corticosteroids alone may improve bone marrow function and facilitate subsequent treatment with cytotoxic agents [10].

Treatment of relapsed/refractory CLL

In patients who are refractory to chlorambucil, or who relapse shortly after an initial response, repeated administration of chlorambucil is often ineffective and the median survival is approximately 15 months [51]. Combination regimens are frequently used in these patients, the most widely used being COP (cyclophosphamide, vincristine and prednisone), CAP (cyclophosphamide, doxorubicin and prednisone) and CHOP (COP + doxorubicin), POACH (cyclophosphamide, doxorubicin, vincristine, cytosine arabinoside and prednisone) and M2 (vincristine, cyclophosphamide, BCNU, melphalan and prednisone) have also been tried. With all these regimens, the response rates are typically 25–35%, with very few CRs, and toxicity is common [44, 47, 51–54].

Randomised trials, mainly in refractory or advanced disease, have failed to show a survival advantage of COP or CHOP over chlorambucil with or without prednisone [36, 45, 46, 55] or of CHOP over chlorambucil with prednisolone [56], despite higher response rates in some studies. A report that stage C patients treated with CHOP survive longer than those treated with COP [57, 58] has not been confirmed by other investigators, and a large overview meta-analysis is currently in progress. In stage C patients, a trial of CHOP versus CHOP with methotrexate failed to show a response or survival difference [56].

It can be argued that trials of CHOP versus chlorambucil test not only the role of the anthracycline, but also the contribution of other drugs in the combination (e.g. cyclophosphamide). For that reason, the MRC CLL3 trial is currently comparing chlorambucil with chlorambucil at the same dose plus epirub-

icin. So far, 300 patients have been randomised, but the results will not be known for some time.

Splenectomy

Splenectomy should be considered if hypersplenism is believed to be the cause of anaemia or thrombocytopenia which has not responded to chemotherapy and corticosteroids. It can induce a prolonged remission in patients whose disease is predominantly localised to the spleen, or in whom a good response to treatment is obtained in all organs except the spleen [15, 59]. If the disease is localised to the spleen, other diagnoses (e.g. splenic lymphoma with villous lymphocytes (SLVL) or mantle-cell lymphoma) should be considered.

Radiation therapy

Radiation therapy is used mainly to reduce enlarged lymph nodes that are painful, unsightly, or compressing vital organs. Low dose total body irradiation (TBI) has been used as a primary therapy for CLL in a few studies. It is no more effective than chemotherapy, and it is frequently associated with severe myelosuppression [60, 61]. In MRC CLL trials 1 and 2, splenic irradiation did not, overall, prove to be superior (or inferior) to cytotoxic drugs [36].

Treatment of infections

Infections are responsible for over half the disease-related deaths in CLL [10]. Most of the infections are bacterial, and pneumonias are their most common manifestation. Mycoses and viral infections also occur. The main cause of infection is hypogammaglobulinaemia. A further cause of infections is alteration of cell-mediated immunity by purine analogues, which, in addition to causing neutropenia by bone marrow toxicity, selectively reduce CD4⁺ lymphocytes. As a result, atypical infections not seen previously in CLL, such as *Pneumocystis carinii* pneumonia, Listeriosis and aspergillosis, have been reported in patients receiving these drugs, most often in non-responders [62–64].

Immunisation is usually ineffective in CLL [65], so antibiotic prophylaxis forms the principle treatment for infections. For most patients, amoxicillin or ampicillin/clavulanic acid, which are active against most upper respiratory tract pathogens, are appropriate. Patients receiving purine analogues should receive prophylaxis for opportunistic infections. For example, the MCR investigators prescribe co-trimoxazole and add oral acyclovir if lymphopenia is marked. Intravenous immunoglobulins have been successful in reducing the frequency of infections in CLL [66].

PURINE ANALOGUES

Mechanisms of action

The purine analogues that have been investigated in CLL are 2'-deoxycoformycin (2-DCF), 2-chlorodeoxyadenosine (2-CDA) and fludarabine. The principle effect of 2-DCF is inhibition of adenosine deaminase, leading to accumulation of deoxyadenosine triphosphate, which inhibits ribonucleotide reductase and inhibits DNA replication and repair [67]. 2-CDA inhibits the synthesis of DNA by inhibiting DNA polymerases and ribonucleotide reductase [68]. Fludarabine inhibits DNA polymerase α , β , γ and ϵ , and DNA ligase, and, following incorporation into DNA, is a highly effective chain terminator [69, 70]. This compound is unique among purine analogues in that it can also inhibit synthesis of RNA [71, 72]. The activity of purine analogues against quiescent cells, which form the

majority of malignant cells in CLL, is a result of disruption of nucleotide pools, which inhibits DNA repair and results in apoptosis [73–75].

Clinical trials with fludarabine

Most clinical trials with fludarabine have been performed in relapsed or refractory CLL [76–84]. The recommended dose is 25 mg/m²/day for 5 days by 30 min intravenous (i.v.) infusion or i.v. push, repeated every 4 weeks. The overall response rates are 40–55%, as high as is achieved with combination regimens, and the CR rates are higher at approximately 13%. Alternative regimens, including once-weekly dosing [83], are less effective.

In previously untreated CLL, fludarabine induced CR in 33% of patients and a nPR in a further 40%, to give a CR rate of 73% according to NCI criteria [30, 84]. This is the highest response rate ever recorded with a single agent in CLL (although the nPR has been considered as "nodular CR" in the MD Anderson studies). In younger patients, the achievement of CR may facilitate further high dose therapy with autologous transplantation procedures.

Fludarabine was generally well tolerated in these trials. The main adverse events were myelosuppression, infections (including some opportunistic infections) and fever of unknown origin. The infections probably resulted partly from the immune dysfunction that occurs in CLL and partly from myelosuppression and depletion of CD4⁺ lymphocytes [62–64]. Reversible pulmonary toxicity has also been reported [85].

The addition of prednisone to fludarabine does not improve response rates, and may increase the risk of opportunistic infections [86, 87]. Combinations of fludarabine with other agents are under investigation, but they are likely to be too toxic unless one compromises by using a lower dose of fludarabine than in single-agent therapy.

A long-term follow-up of patients treated with fludarabine alone was published recently [30]. Younger age, a smaller number of prior treatments, and a CR or nPR were associated with longer survival. Median time to progression after a response was 33 months in previously untreated patients and 22 months in those previously treated.

In a recent trial at the Royal Marsden Hospital, fludarabine was evaluated in 52 previously treated patients (D. Catovsky, unpublished observations). Most of the patients were refractory to chlorambucil and 12 had CLL-PL. The response rates are summarised in Table 3. Patients who achieved a CR or a PR had significantly greater survival than those who did not respond or were not evaluable (Figure 3). The time to disease progression in responders was not significantly different between CLL and CLL-PL (Figure 4).

Preliminary results from a comparative trial of fludarabine against CAP [82] and comparisons with previous trials [30] suggest that fludarabine induces slightly longer responses than

Table 3. Response rates to fludarabine in the Royal Marsden Hospital series (D. Catovsky, unpublished observations)

| Disease type | No. of cases | CR (%) | PR (%) | CR + PR (%) |
|--------------|--------------|--------|---------|-------------|
| CLL | 34* | 6 (18) | 14 (41) | 20 (59) |
| CLL-PL | 12 | – | 9 (75) | 9 (75) |
| Overall | 46 | 6 (13) | 23 (50) | 29 (63) |

*Excludes 6 patients who died within 2 months and were not evaluable.

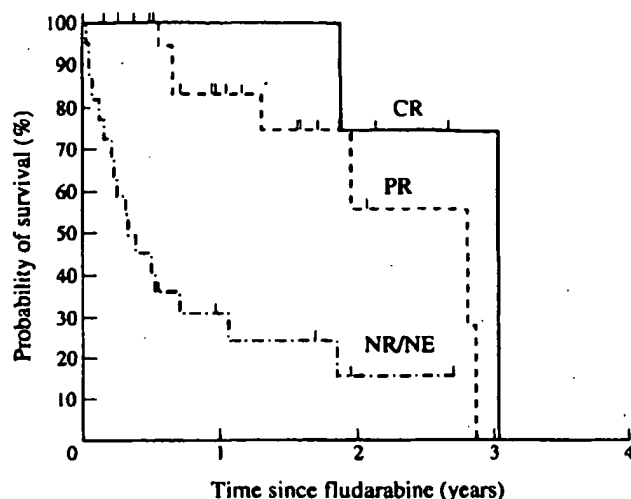


Figure 3. Survival of patients with CLL or CLL-PL ($n = 46$) (see Figure 4) by response to fludarabine in the Royal Marsden Hospital series (D. Catovsky, unpublished observations). CR, complete response; PR, partial response; NR/NE, no response/not evaluable. $\chi^2 = 19.34$, $df = 2$, $P < 0.005$.

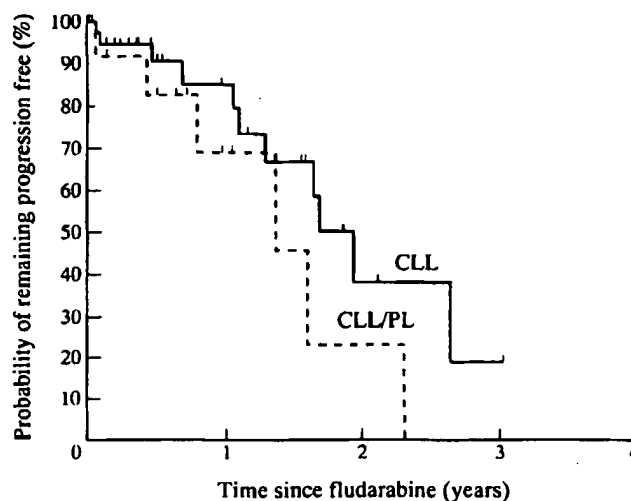


Figure 4. Probability of disease progression in patients in the Royal Marsden Hospital series (D. Catovsky, unpublished observations). CLL, chronic lymphocytic leukaemia; CLL-PL, CLL with increased prolymphocytes.

combination regimens, but the long-term survival is similar. Randomised trials are ongoing to clarify the effects of fludarabine on long-term survival, and determine whether it should be reserved for second-line treatment or used as first-line therapy.

Other purine analogues

Other purine analogues appear to be less promising than fludarabine in the treatment of CLL. 2-CDA induces response rates of 45–55% in previously treated patients, but CRs are less common than with fludarabine and are of shorter duration [88–90]. Myelosuppression and infection are the principal adverse events. 2-DCF induces response rates of 20–27%, with few CRs, and has been associated with opportunistic infections [91, 92].

IFN- α

IFN- α has been reported to reduce the lymphocyte counts in early CLL [93–97], but the responses are transient and no CRs have been observed. IFN- α is not effective in advanced disease [98]. The activity of IFN- α might be more appropriately tested in patients in remission, to see whether it can prolong the remission or improve survival.

HAEMATOPOIETIC GROWTH FACTORS

Recombinant granulocyte colony stimulating factor reduces neutropenia and infections in patients with non-Hodgkin's

lymphoma receiving CHOP [99, 100], allowing the dose to be maintained or increased [101, 102]. Clinical trials are required to determine whether a similar effect could be achieved in patients with CLL treated with fludarabine.

BONE MARROW TRANSPLANTATION

Allogeneic and autologous BMT have recently been attempted in small groups of patients with poor prognosis CLL [103–107]. Various regimens, including TBI, CHOP and fludarabine, were used to induce a CR before transplantation. The results of three trials are summarised in Table 4 [103, 106, 107]. CRs, some of which were durable, were obtained in some patients. The main causes of death were relapse, graft-versus-host disease and toxicity of the conditioning regimen. These results suggest that it is possible to undertake BMT in patients with poor prognosis CLL. Favourable responses were obtained in some patients aged over 60 years. This indicates that clinical trials of BMT in CLL need not necessarily be restricted to younger patients.

MONOCLONAL ANTIBODIES

Monoclonal antibodies against B-cell antigens, either alone or combined with toxins or radioisotopes, have been attempted in a few patients [108]. The published results have been largely disappointing, probably because the antibodies cannot reach malignant cells in the bone marrow and lymph nodes, and

Table 4. Results of BMT in CLL in three recent trials

| First author [Ref.] | No. of patients | Median age (years) | Type of transplant | % CR | % projected 2 year survival |
|------------------------------|-----------------|--------------------|------------------------------------|------------------------------------|------------------------------------|
| Michallet [104] | 17 | 40 | Allogeneic | 88 | – |
| Rabinowe <i>et al.</i> [108] | 20 | 40 | Autologous (12) Syngeneic (8) | Almost 90* | 60 |
| Khoury <i>et al.</i> [107] | 22 | 47.5 | Allogeneic (11) Autologous (11) | Allogeneic (64) Autologous (55) | Allogeneic (90) Autologous (40) |

*Documented by phenotype and Ig gene rearrangements.

because the malignant cells are capable of antigenic modulation. The humanised monoclonal CAMPATH 1H antibody, given subcutaneously, has shown promising results at our institution in patients with treatment-resistant CLL and B-prolymphocytic leukaemia (B-PLL) (D. Catovsky and M. Dyer, unpublished observations).

TREATMENT OF "CLL VARIANTS"

A number of B-cell disorders have been vaguely described as "CLL variants". The only disease closely related to CLL is CLL-PL, which consists of a CLL background with an increase in the prolymphocyte population. CLL-PL is a subtle transformation of CLL arising from the same clone, with a higher proliferation rate assessed by monoclonal antibody Ki-67 labelling and evidence of trisomy 12 in approximately half the cases [21].

Richter's syndrome is a transformation of CLL to a large-cell lymphoma [109]. Recent evidence indicates that half the cases of Richter's syndrome develop from the same CLL clone and the rest from a new B-cell clone [110]. The prognosis is poor, with median survival of 4–6 months. Combination regimens (e.g. CHOP) are the treatment of choice. The prognosis is strongly dependent on whether a remission can be obtained.

Other diseases, such as B-PLL, hairy cell leukaemia (HCL) and SLVL are distinct diseases [111]. CLL-PL and B-PLL seem to benefit from fludarabine [112–114], whereas HCL is treated with 2-CDA or 2-CDF. The treatment of choice for SLVL is splenectomy, although a minority of patients respond to fludarabine.

CONCLUSION

The introduction of new treatments, particularly the purine analogues, has stimulated renewed interest in CLL. The high efficacy and good overall tolerability of these agents raises the possibility of improved long-term survival, and the high CR rate allows BMT to be considered in younger patients. This represents a shift of emphasis in the treatment of CLL from palliative therapy to curative intent. Controlled clinical trials, with carefully identified subgroups of patients and rigorous diagnosis and assessment criteria, are required to define the place of new treatments and improve the outcome of this disease.

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Oral Session

Chronic Lymphocytic Leukemia - Therapy, Excluding Transplantation

**Immunochemotherapy with Fludarabine (F),
Cyclophosphamide (C), and Rituximab (R)
(FCR) Versus Fludarabine and
Cyclophosphamide (FC) Improves Response
Rates and Progression-Free Survival (PFS) of
Previously Untreated Patients (pts) with
Advanced Chronic Lymphocytic Leukemia
(CLL)**

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Abstract

Introduction: Previous phase II studies have suggested that a combination of FCR may increase the outcome of both untreated and relapsed CLL pts. In order to validate this concept the German CLL study group (GCLLSG) initiated a multicentre, multinational phase III trial, CLL8, to evaluate the efficacy and tolerability of FCR versus FC for the first-line treatment of pts with advanced CLL.

Methods and Patients: 817 pts with good physical fitness as defined by a cumulative illness rating scale (CIRS) score (Extermann et al., JCO 1998) of up to 6 and a creatinine clearance (cr cl) \geq 70 ml/min were enrolled between July 2003 and March 2006. Pts were randomly assigned to receive 6 courses of either FC (N=409; F 25mg/m² i.v. d1–3 and C 250 mg/m² i.v. d1–3; q 28 days) or FC plus R (N=408; 375 mg/m² i.v. d 0 at first cycle and 500 mg/m² d1 all subsequent cycles; q 28 days). Both treatment arms were well balanced with regard to age, stage, genomic aberrations and VH status. 64% were Binet B, 32% Binet C and 5% Binet A. The median age was 61 years (range 30 to 81), the median CIRS score was 1 (range 0–8). The overall incidences of trisomy 12 and abnormalities of 13q, 11q23, and 17p13 detected by FISH were 12%, 57%, 25%, and 8%, respectively, with no statistically significant differences between treatment arms. A

mean number of 5.2 courses was given in the FCR arm versus 4.8 courses in the FC arm ($p=0.006$). 74% (FCR) and 67% (FC) of pts received 6 cycles. Dose was reduced by more than 10% in at least one treatment course in 43% (FCR) and 30% (FC) of pts, and in 21% (FCR) and 17% (FC) of all treatment courses given. 17 pts did not receive any study medication, 10 due to violation of enrolment criteria (4 decreased renal function, 2 active secondary malignancies, 2 active infections, 1 autoimmune thrombocytopenia, 1 pt not requiring treatment), 3 due to withdrawal of consent, 2 due to worsened concomitant diseases. 2 pts were lost before start of treatment. 56 pts were not evaluable for response: 17 did not receive any study medication, 16 withdrew consent before interim staging, 7 due to violation of enrolment criteria, 4 discontinued treatment due to toxicity and 12 due to early death (caused by toxicity, progression or secondary malignancy). Prophylactic use of antibiotics or growth factors was not generally recommended in the protocol.

Results: At the time of analysis, June 2008, the median observation time was 25.5 months (mo). 761 pts (FCR 390; FC 371) were evaluable for response, 787 pts (FCR 400; FC 387) for PFS and all for OS. The overall response rate (ORR) was significantly higher in the FCR arm (95%; 370/390) compared to FC (88%; 328/371 ($p=0.001$)). The complete response rate of the FCR arm was 52% as compared to 27.0% in the FC arm ($p<0.0001$). PFS was 76.6% at 2 years in the FCR arm and 62.3% in the FC arm ($p<0.0001$). There was a trend for an increased OS rate in the FCR arm (91% vs 88% at 2 years $p=0.18$). Hazard Ratio for PFS was 0.59, for OS 0.76. The largest benefit for FCR was observed in Binet stage A and B with regard to CR, ORR and PFS (A: $p=0.01$, B: $p<0.0001$). FCR treatment was more frequently associated with CTC grade 3 and 4 adverse events (47% of FC vs 62% of FCR treated pts). Severe hematologic toxicity occurred in 55% (FCR) versus 39% (FC) of all patients. Significant differences were observed for neutropenia (FCR 33,6%; FC 20,9% $p=0.0001$) and leukocytopenia (FCR 24%; FC 12,1% $p<0.0001$) but not for thrombocytopenia (FCR 7,4%; FC 10,8% $p=0.09$) and anemia (FCR: 5,4% FC 6,8% $p=0.42$). The incidence of CTC grade 3 or 4 infections was not significantly increased in the FCR arm (18,8% versus 14,8% in the FC arm, $p=0.68$). Tumor lysis syndrome (FCR 0,2% FC 0,5%) and cytokine release syndrome (FCR 0,2% FC 0,0%) were rarely observed in both arms. Treatment related mortality occurred in 2.0% in the FCR and 1.5% in the FC arm. Multivariate analyses were performed to evaluate factors predicting outcome. Amongst these variables age, sex, Binet stage, CIRS score, renal function ($cr\ cl < 70\ ml/min$) were independent prognostic factors predicting OS or PFS.

Conclusion: Treatment with FCR chemoimmunotherapy improves response rates and PFS when compared to

the FC chemotherapy. FCR caused more neutropenia/leukopenia without increasing the incidence of severe infections. These results suggest that FCR chemoimmunotherapy might become the new standard first-line treatment for physically fit CLL patients.

Footnotes

Corresponding author

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Late-Breaking Abstracts

Late-Breaking Abstracts

**Rituximab, Fludarabine, and
Cyclophosphamide (R-FC) Prolongs
Progression Free Survival in Relapsed or
Refractory Chronic Lymphocytic Leukemia
(CLL) Compared with FC Alone: Final
Results from the International Randomized
Phase III REACH Trial**

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Abstract

The addition of rituximab to a variety of chemotherapy regimens for the treatment of patients with CLL has yielded promising results in phase II trials. The R-FC regimen demonstrated particularly high rates of overall response (ORR), complete remission (CR), progression-free survival (PFS) and overall survival (OS) in relapsed/refractory CLL (Wierda, et al, JCO 2005). REACH was an open-label, multicenter, randomized, phase III study to evaluate the efficacy and tolerability of R-FC versus FC in relapsed or refractory patients with CD20 positive CLL. The primary endpoint of the study was progression free survival.

Five hundred and fifty two patients from 17 countries were randomized (1:1) to receive either R-FC or FC. Rituximab was administered IV before the FC infusion for a total of 6 treatment cycles at intervals of 28 days (Cycle 1: 375 mg/m² IV; Cycles 2–6: 500 mg/m² IV). Fludarabine (25 mg/m² IV/day) and cyclophosphamide (250 mg/m² IV/day) were administered over 3 days for 6 cycles. Baseline demographics, disease characteristics, and prognostic factors were well balanced between the two arms. Median age was 63 years. All Binet stages were represented (A 10%, B 59%, C 31%). A median of one prior treatment had been administered, consisting of single-agent alkylator therapy (66%), purine-analogs (16%), or combination treatments (CHOP, COP, F-containing, 18%). Patients with prior FC combination treatment or prior rituximab were not eligible. Median observation time was 25 months.

The primary endpoint PFS was significantly prolonged by median 10 months in the R-FC arm (30.6 months) compared to FC (20.6 months, $p = 0.0002$, Hazard Ratio (HR) 0.65 [95% CI 0.51; 0.82]). Secondary endpoints EFS, TTNT, DR showed similar results. ORR were higher for R-FC vs. FC (70% vs. 58%, $p = 0.0034$), due to superior CR rates (24% vs. 13%, $p = 0.0007$). Multiple subgroups were analyzed applying a

Cox-regression model: all Binet stages experienced similar incremental benefits in PFS (HR Binet A 0.75, B 0.65, C 0.61). Mutational status and cytogenetic subgroups remained prognostic and benefited from the addition of rituximab to FC (HR IgVH unmutated 0.62, mutated 0.7; del17p pos 0.75, neg 0.63; del13q pos 0.56, neg 0.77). Median overall survival was not reached for R-FC and was 53 months for FC, (p=0.29, HR 0.83). Of 47 patients that relapsed and were treated in the R-FC arm, 30% received rituximab again. Sixty-nine patients were treated at relapse in the FC arm, and 49% received rituximab.

Grade 3/4 Adverse Events were higher in the R-FC arm (80%) vs. FC (74%), but serious adverse events were similar (50% vs. 48%, respectively). Grade 3/4 neutropenia and febrile neutropenia were only marginally increased for R-FC (42% and 15%) vs. FC (40% and 12%, respectively), the same was seen for thrombocytopenia (R-FC 11% vs. FC 9%). Grade 3/4 infections (R-FC 18%, FC 19%) were similar, and there was no difference in bacterial, viral, or fungal infections between the two arms. Grade 3/4 anemia was slightly increased in the FC arm (R-FC 2%, FC 5%). Slightly higher Fatal Adverse Events were seen with R-FC (13%) vs. FC (10%). Fatal SAEs were mainly due to infections, secondary neoplasms, and cardiac disorders. Summary and conclusion: In this large randomized trial in relapsed or refractory CLL, with 10 months improvement in PFS and a doubling of CR rates, R-FC was statistically significant and clinically meaningful superior to FC in the primary analysis. Improvement in PFS was seen across most subgroups, including all Binet stages. Fatal AEs were relatively high in both arms. However, overall, the addition of rituximab to FC in REACH showed a very favorable risk-benefit profile and did not reveal any new or unexpected safety signals.

Footnotes

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Speakers Bureau; *Santaris Pharma*: Consultancy; *Celgene*: Consultancy; *Fresenius*: Consultancy. **Montillo**: *Bayer Schering*: Honoraria, Research Funding, Speakers Bureau; *Mundipharma*: Honoraria. **Ganly**: *Novartis*: Consultancy; *Roche*: Consultancy. **Rosta**: *Roche*: Honoraria. **Mendila**: *Roche*: Employment. **Maurer**: *Roche*: Employment. **Wenger**: *Roche*: Employment.

Basel, 27 February 2009

MabThera approved in the EU for patients with chronic lymphocytic leukaemia **New standard of care available for patients living with previously-untreated disease**

Roche announced today that the European Commission has approved MabThera (rituximab) in combination with chemotherapy for use in patients with previously-untreated chronic lymphocytic leukaemia (CLL), the most common type of leukaemia to affect adults. The approval is based on compelling results from the pivotal phase III study CLL8.

"Data from the CLL8 trial suggest that MabThera used in combination with chemotherapy has the potential to become the standard of care for the treatment of CLL", said Professor Michael Hallek, University of Cologne, Germany, who led the German CLL Study Group (GCLLSG) in conducting the CLL8 trial. "Today's approval will make the best treatment, MabThera plus chemotherapy, available to patients with CLL across Europe".

The results showed that patients receiving MabThera in combination with chemotherapy as first-line treatment lived an average of 40 months without their cancer progressing compared to an average of 32 months for patients receiving chemotherapy alone.¹ At present, CLL is considered incurable and the aim of treatment is to control the disease by managing symptoms and extending the time patients live without their disease worsening. The results of CLL8 demonstrate that patients treated with MabThera lived longer without their disease progressing, reducing the number of frequent hospital visits.

"Today's approval of MabThera in CLL is great news for patients suffering from this devastating disease. We have seen MabThera transformed the lives of more than 1.5 million patients with non-Hodgkin's lymphoma (NHL) and we hope this approval will bring about a similar change for people suffering from this condition", said William M. Burns, CEO, Roche Pharmaceuticals Division.

CLL is the most common type of leukaemia in adults, accounting for approximately 30-40% of all forms of leukaemia in Western countries.² Overall incidence of CLL is around three per 100,000³ and is 30% more common in men than women.² It mainly affects the elderly with 70-80% of patients diagnosed after the age of

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55³ and it is most commonly diagnosed between 65 and 70 years of age.³ While CLL is generally considered a disease that is slow to progress, a significant proportion of patients have rapidly progressing forms of the disease.³

Earlier this year Roche submitted a Marketing Authorisation Application (MAA) to the European Medicines Agency (EMA) for the use of MabThera in patients who have previously been treated for their CLL but whose cancer has returned.

About CLL8

The CLL8 study is an international study conducted by the German CLL Study Group and Professor Michael Hallek (University Hospital Cologne, Germany) in collaboration with Roche. It included 817 patients with CLL receiving first-line treatment. The study was conducted at 191 study sites across 11 countries. In this randomised study, patients received either MabThera in combination with chemotherapy (fludarabine and cyclophosphamide) or chemotherapy alone. The primary endpoint of the study was progression-free survival. No new or unexpected safety signals were observed.

About MabThera

MabThera is a therapeutic antibody that binds to a particular protein - the CD20 antigen - on the surface of normal and malignant B-cells. It then recruits the body's natural defences to attack and kill the marked B-cells. Stem cells (B-cell progenitors) in bone marrow lack the CD20 antigen, allowing healthy B-cells to regenerate after treatment and return to normal levels within several months.

In oncology, MabThera is indicated:

- For the treatment of previously untreated patients with stage III-IV follicular lymphoma in combination with chemotherapy
- As maintenance therapy for patients with relapsed/refractory follicular lymphoma responding to induction therapy with chemotherapy with or without MabThera
- For the treatment of patients with CD20 positive diffuse large B cell non-Hodgkin's lymphoma in combination with CHOP (cyclophosphamide, doxorubicin, vincristine, prednisolone) chemotherapy
- As monotherapy for treatment of patients with stage III-IV follicular lymphoma who are chemoresistant or are in their second or subsequent relapse after chemotherapy.

In addition, in rheumatology, MabThera in combination with methotrexate, is indicated for the treatment of adult patients with severe active rheumatoid arthritis who have had an inadequate response or intolerance to other disease-modifying anti-rheumatic drugs (DMARD) including one or more tumour necrosis factor (TNF) inhibitor therapies.

MabThera is known as Rituxan in the United States, Japan and Canada. To date, more than 1.5 million patients have been treated with MabThera worldwide.

Genentech and Biogen Idec co-market Rituxan in the United States, and Roche markets MabThera in the rest of the world, except Japan, where MabThera is co-marketed by Chugai and Zenyaku Kogyo Co. Ltd.

About Roche

Headquartered in Basel, Switzerland, Roche is one of the world's leading research-focused healthcare groups in the fields of pharmaceuticals and diagnostics. As the world's biggest biotech company and an innovator of products and services for the early detection, prevention, diagnosis and treatment of diseases, the Group contributes on a broad range of fronts to improving people's health and quality of life. Roche is the world leader in in-vitro diagnostics and drugs for cancer and transplantation, and is a market leader in virology. It is also active in other major therapeutic areas such as autoimmune diseases, inflammatory and metabolic disorders and diseases of the central nervous system. In 2008 sales by the Pharmaceuticals Division totalled 36.0 billion Swiss francs, and the Diagnostics Division posted sales of 9.7 billion francs. Roche has R&D agreements and strategic alliances with numerous partners, including majority ownership interests in Genentech and Chugai, and invested nearly 9 billion Swiss francs in R&D in 2008. Worldwide, the Group employs about 80,000 people. Additional information is available on the Internet at www.roche.com.

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■ Medical News from ASH: Hematology: American Society of Hematology Meeting

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ASH: Rituximab for CLL Should Change Practice

By Michael Smith, North American Correspondent, MedPage Today
Published: December 11, 2008
Reviewed by Robert Jasmer, MD; Associate Clinical Professor of Medicine,
University of California, San Francisco .

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SAN FRANCISCO, Dec. 11 —
Combining the monoclonal antibody
rituximab (Rituxan) with standard
chemotherapy for chronic lymphocytic
leukemia should become the new
standard of care, researchers said
here.

Two major clinical trials presented at
the American Society of Hematology
meeting — both claiming to be the
largest ever in their patient groups —
found that adding the immune
modulator to chemotherapy markedly
improved response rates compared
with chemotherapy alone.

"In my opinion, this is going to change
practice," said Michael Hallek, M.D., of
the University of Cologne in Cologne, Germany, who led a study of 817 previously untreated
patients.



Michael Hallek, M.D.
University of Cologne, Cologne, Germany

Action Points

- Explain to interested
patients that standard

Concurring was Tadeusz Robak, M.D., Ph.D., of Medical
University in Lodz, Poland, who led a study that enrolled
552 patients with refractory or relapsing disease.

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therapy for chronic lymphocytic leukemia (CLL) is chemotherapy.

- Note that these studies show that adding the monoclonal antibody rituximab (Rituxan) to the mix improves response rates.
- Note that these studies were published as abstracts and presented orally at a conference. These data and conclusions should be considered to be preliminary until published in a peer-reviewed journal.

"We now have enough evidence to say that immunochemotherapy with rituximab, purine analogues, and cyclophosphamide is better both in untreated and difficult to treat patients," Dr. Robak said.

The two trials were large randomized controlled studies and so meet the gold standard needed to convince physicians, said Linda Burns, M.D., of the University of Minnesota in Minneapolis, who moderated the press conference at which the studies were presented.

"I would anticipate this will be practice-changing," Dr. Burns said.

Dr. Hallek said he expects a new indication for rituximab within a few months, and after that, "it should become the new global standard."

All this enthusiasm is based on the outcomes of comparisons of regimens based on fludarabine (Fludara) and cyclophosphamide, with or without rituximab.

Chronic lymphocytic leukemia, the most common form of the disease in adults, has an incidence of about 15,000 cases a year in the U.S. and causes about 4,500 deaths annually.

Dr. Hallek and colleagues found that among previously untreated patients the complete response rate for the rituximab regimen was 44.5%, compared with 22.9% for chemotherapy alone. The difference was significant at $P < 0.01$.

At the same time, the partial response rate was lower — 3.3% versus 8.1% — probably because more patients achieved a complete response, he said. The difference was also significant at $P < 0.01$.

After a median observation time of 25.5 months, the median progression-free survival was 42.8 months for the rituximab regimen, compared with 32.3 months for chemotherapy alone. The difference was significant at $P = 0.000007$.

The rituximab regimen was associated with more neutropenia than the chemotherapy, but did not cause more infections or other severe side effects, Dr. Hallek said.

Dr. Robak and colleagues — in the so-called REACH trial of patients with refractory or relapsing disease — found a complete response rate of 24.3% for the rituximab regimen, compared with 13% for chemotherapy alone. The difference was significant at $P = 0.0007$.

Partial response rates were similar and the overall response rates were 69.9% for rituximab and 58% for chemotherapy alone, a difference that was significant at $P = 0.0034$.

After a median observation time of 25.3 months, the median progression-free survival was 30.6 months for the rituximab regimen, compared with 20.6 months for chemotherapy alone. The difference was significant at $P = 0.0002$.

Safety was in line with previous studies, he said, with a slight increase in febrile neutropenia, infusion reactions, benign and malignant neoplasms, and hepatitis B for the rituximab regimen.

Neither researcher was able yet to say how the new regimen will affect survival.

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Both studies were supported by Roche and Dr. Hallek reported research support and consultancy fees from the company.

Dr. Robak reported consultancy fees, research funding, and/or honoraria from Celgene, Roche, Genmab, Cambridge Antibody Technology and GlaxoSmithKline.

Primary source: Blood

Source reference:

allek M, et al "Immunochemotherapy with fludarabine (F), cyclophosphamide (C), and rituximab (R) (FCR) versus fludarabine and cyclophosphamide (FC) improves response rates and progression-free survival (PFS) of previously untreated patients (pts) with advanced chronic lymphocytic leukemia (CLL)" *Blood* 2008; 112(11): Abstract 325.

Additional source: Blood

Source reference:

Robak T, et al "Rituximab, fludarabine, and cyclophosphamide (R-FC) prolongs progression-free survival in relapsed or refractory chronic lymphocytic leukemia (CLL) compared with FC alone: Final results from the International Randomized Phase III REACH Trial" *Blood* 2008; 112(11): Abstract LBA-1.

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